REMARKS/ARGUMENTS

Claims 28-32 are pending in this application.

I. Claim Rejections Under 35 U.S.C. §101 and 35 U.S.C. §112, First Paragraph

Claims 28-32 remain rejected under 35 U.S.C. §101 because the claimed invention allegedly "is not supported by either a specific and substantial asserted utility or a well established utility." (Page 2 of the instant Office Action).

Claims 28-32 are also rejected under 35 U.S.C. §112, first paragraph, because allegedly "the claimed invention is not supported by either a credible, specific and substantial asserted utility or a well established utility," and therefore "one of skill in the art clearly would not know how to use the claimed invention." (Page 10 of the instant Office Action).

For the reasons outlined below, Applicants respectfully disagree and traverse the rejection. With respect to Claims 28-32, Applicants submit that not only has the Patent Office not established a *prima facie* case for lack of utility and enablement, but that the PRO1303 polypeptide and the claimed antibodies that bind it possess a credible, specific and substantial asserted utility and are fully enabled.

A. The results of the gene amplification assay provide utility for the PRO1303 polypeptide and antibodies that bind it

First of all, Applicants respectfully maintain the position that the specification discloses at least one credible, substantial and specific asserted utility for the PRO1303 polypeptide and the claimed antibodies that bind it for the reasons previously set forth in Applicants' Responses filed on December 21, 2004, and July 5, 2005.

Furthermore, as discussed in Applicants' Response of July 5, 2005, Applicants rely in part on the gene amplification data for patentable utility of the antibodies to the PRO1303 polypeptide, and the gene amplification data for the gene encoding the PRO1303 polypeptide is clearly disclosed in the instant specification under Example 143. The specification discloses that the nucleic acids encoding PRO1303 had ΔCt value of > 1.0, which is a **more than 2-fold increase**, for primary lung tumors LT13, LT15, LT16; for lung cell line A549; and for the

primary colon tumor CT16. Accordingly, the present specification clearly discloses strong evidence that the gene encoding the PRO1303 polypeptide is significantly amplified in a significant number of lung and colon tumors.

In further support, Applicants have submitted, in their Response filed July 5, 2005, a Declaration by Dr. Audrey Goddard. Applicants particularly draw the Examiner's attention to page 3 of the Goddard Declaration which clearly states that:

> It is further my considered scientific opinion that an at least **2-fold increase** in gene copy number in a tumor tissue sample relative to a normal (*i.e.*, non-tumor) sample is significant and useful in that the detected increase in gene copy number in the tumor sample relative to the normal sample serves as a basis for using relative gene copy number as quantitated by the TaqMan PCR technique as a diagnostic marker for the presence or absence of tumor in a tissue sample of unknown pathology. Accordingly, a gene identified as being amplified at least 2-fold by the quantitative TaqMan PCR assay in a tumor sample relative to a normal sample is **useful as a marker for the diagnosis of cancer**, for monitoring cancer development and/or for measuring the efficacy of cancer therapy. (Emphasis added).

As indicated above, the gene encoding the PRO1303 polypeptide shows at least a two fold amplification in five different lung and colon tumors and tumor cell lines. In addition, the Goddard Declaration clearly establishes that the TaqMan real-time PCR method described in Example 143 has gained wide recognition for its versatility, sensitivity and accuracy, and is in extensive use for the study of gene amplification. The facts disclosed in the Declaration also confirm that based upon the gene amplification results, one of ordinary skill would find it credible that PRO1303, and hence the claimed antibodies that bind it, are diagnostic markers of lung and colon cancer.

The Examiner asserts that the arguments concerning gene amplification are not persuasive in overcoming the rejections because "the PRO1303 DNA's utility is not being questioned in the instant rejections." (Page 13 of the instant Office Action). The Examiner maintains that an increased copy number of DNA does not necessarily result in increased levels of expression of the polypeptide.

Applicants have previously submitted ample evidence to show that, in general, if a gene is amplified in cancer, it is more likely than not that the encoded protein will be expressed at an elevated level. First, the articles by Orntoft *et al.*, Hyman *et al.*, and Pollack *et al.*, (made of record in Appellants' Response filed July 5, 2005) collectively teach that in general, gene amplification increases mRNA expression. Second, the Declaration of Dr. Paul Polakis, principal investigator of the Tumor Antigen Project of Genentech, Inc., the assignee of the present application, shows that, in general, there is a correlation between mRNA levels and polypeptide levels.

The Examiner asserts that "[f]rom the uncertainty in the cited art concerning the correlation between gene amplification and gene product overexpression, one of ordinary skill in the art would doubt the alleged utility because in the absence of information showing the correlation, one would doubt that the correlation exists." (Page 14 of the instant Office Action). In particular, the Examiner asserts that the cited art (specifically, the Pollack *et al.* paper) showing that 62% of highly amplified genes show moderately or highly elevated expression "very much shows the uncertainty in the art concerning the correlation of DNA copy number and RNA expression." (Page 15 of the instant Office Action). The Examiner further asserts that the data cited in the Polakis Declaration showing that 80% of the time there was a correlation between mRNA and corresponding protein levels "shows the uncertainty in the art whether there is any correlation between overexpressed RNA and overexpressed protein." (Page 15 of the instant Office Action). The Examiner concludes that the actual level of correlation is 62% multiplied by 80%, or about 50%.

Applicants respectfully submit that Pollack *et al.* state that "we are likely underestimating the contribution of DNA copy number changes to altered gene expression" (page 12967, col. 2), indicating that the 62% number cited by the Examiner is most probably an underestimate. This conclusion is consistent with the results presented by Orntoft *et al.*, who found that the level of correlation between DNA copy number and increased mRNA levels was from **77-80%** (page 40, col. 2). Orntoft *et al.* also found a "highly significant" correlation between mRNA and protein

levels, with the two data sets studied having correlations of 39/40 (98%) and 19/26 (73%). This data is consistent with the 80% correlation noted in the Polakis Declaration.

Applicants note that the Hyman reference "found 44% of highly amplified genes showing overexpression at the mRNA level. However, in the more detailed discussion of their results, Hyman *et al.* teach that "[u]p to 44% of the highly amplified transcripts (CGH ratio, >2.5) were overexpressed (***i.e.*, belonged to the global upper 7% of expression ratios**) compared with only 6% for genes with normal copy number." (See page 6242, col. 1; emphasis added). These details make it clear that Hyman *et al.* set a highly restrictive standard for considering a gene to be overexpressed; yet almost half of all highly amplified genes met even this highly restrictive standard. Therefore, the analysis performed by Hyman *et al.* clearly shows that "it is more likely than not" that a gene which is amplified in tumor cells will have increased gene expression.

Applicants also respectfully point out that the Examiner's calculation of DNA/protein correlation by taking the product of the (underestimated) DNA/mRNA correlation and the mRNA/protein correlation is not valid because not all mRNA amplification is the result of gene copy number increases. Transcripts that are elevated due to other mechanisms may be less likely to result in increased protein levels, and thus would bring down the average correlation. Even if this calculation is accurate, Applicants respectfully note that taking a more accurate correlation for DNA/mRNA of 79%, as observed by Orntoft *et al.*, leads to an overall correlation for DNA with protein levels of 63%, which is certainly more likely than not.

Accordingly, as discussed in Applicants' Response filed July 5, 2005, since the standard is not absolute certainty, a *prima facie* showing of lack of utility has not been made in this instance and the burden to provide further evidence of utility has not shifted to Applicants.

Taken together, although there are some examples in the scientific art that do not fit within the central dogma of molecular biology that there is a correlation between polypeptide and mRNA levels, with the Examiner citing Pennica *et al.* and Konopka *et al.*, these instances are exceptions rather than the rule. In the vast majority of amplified genes, the teachings in the art, as exemplified by Orntoft *et al.*, Hyman *et al.*, Pollack *et al.*, and the Polakis declaration,

overwhelmingly show that gene amplification influences gene expression at the mRNA and protein levels.

Thus, one of skill in the art would reasonably expect in this instance, based on the amplification data for the PRO1303 gene, that the PRO1303 protein is concomitantly overexpressed. Accordingly, one skilled in the art at the time the application was filed would have understood that the claimed PRO1303 polypeptide and the claimed antibodies that bind it had utility in, for example, the diagnosis of cancer, and would have known exactly how to use the claimed antibodies without any undue experimentation.

B. <u>The results of the adipocyte glucose/FFA uptake assay provide utility for the PRO1303 polypeptide and antibodies that bind it</u>

Applicants further rely in part on the adipocyte glucose/FFA uptake assay (Example 149, Assay #94) for support of patentable utility. The adipocyte glucose/FFA uptake assay is designed to determine whether a polypeptide is capable of modulating, either positively or negatively, the uptake of glucose or free fatty acids in adipocyte cells. By making such determinations, the assay identifies polypeptides that are expected to be useful for treating disorders wherein stimulation or inhibition of glucose uptake by adipocytes is expected to be therapeutically effective. Examples of these types of disorders include obesity, diabetes, and hyper- or hypo-insulinemia.

PRO1303 was demonstrated to be a stimulator of glucose/FFA uptake in this assay. As discussed in Applicants' Response filed July 5, 2005, it was known in the art at the time of filing that agents which increased glucose uptake, such as troglitazone and pioglitazone, were useful in the treatment of diabetes. Treatment with vanadium salts, another agent which increased glucose uptake, was shown to lower glucose levels in hyperglycemic rats. Diabetes, hyperglycemia, and obesity were know at the time of filing to be closely linked conditions (see, for example, Sandouk, page 352). Thus one of skill in the art would have understood that stimulators of glucose uptake would be useful in the treatment of diabetes, obesity, and hyperglycemia. Accordingly, a variety of real-life utilities, such as treatments for glucose uptake related diseases,

including obesity and diabetes, are envisioned for PRO1303 based on the glucose/FFA uptake assay results disclosed herein.

The Examiner asserts that "the specification does not actually describe whether PRO1303 actually increases glucose uptake because the assay results were set forth as being positive for glucose and/or FFA uptake. If PRO1303 was positive for only FFA uptake, then the arguments concerning utility based on glucose uptake are not persuasive." (Page 16 of the instant Office Action).

Applicants respectfully point out that it was well known in the art at the time of filing that both glucose and FFA levels were associated with diabetes, obesity, and hyperinsulinemia. In fact, FFA levels are one of the factors which regulate glucose uptake. As discussed in Applicants' Response filed July 5, 2005, Fabris *et al.* (made of record by the Examiner in the Office Action mailed April 5, 2005) explains that high circulating FFA levels lead to FFA-induced insulin resistance (page 604, col. 2). This resistance leads to less utilization of glucose, which contributes to the development of obesity or diabetes. Santomauro *et al.* (made of record by the Examiner in the Office Action mailed April 5, 2005) demonstrated that lowering plasma FFA levels increases insulin sensitivity, and thus increases glucose uptake in response to insulin.

The Examiner asserts that "the applicant fails to show that uptake of FFA is a hallmark of therapeutically effective agents to treat the indicated diseases." (Page 16 of the instant Office Action). In fact, the reference by Santomauro *et al.*, cited by the Examiner in the previous Office Action, confirms that "lowering of elevated plasma FFA levels can reduce insulin resistance/hyperinsulinemia and improve oral glucose tolerance in lean and obese nondiabetic subjects and in obese patients with type 2 diabetes" (Abstract). Thus it is clear that agents which decrease circulating FFA levels are effective in the treatment of diseases such as obesity and diabetes.

It was further known in the art at the time of filing that antidiabetic agents such as the thiazolidinediones (including troglitazone and pioglitazone) discussed in Applicants' Response filed July 5, 2005 as increasing glucose uptake, also increase FFA uptake by adipocytes. For example, Frohnert *et al.* (J. Biol. Chem. 272:3970-3977 (1999); copy enclosed as Exhibit 1)

found that troglitazone stimulates increased expression of fatty acid transport protein (FATP) in adipocytes by 3-fold (page 3974, col. 1; Fig. 5). Troglitazone was also found to significantly increase uptake of fatty acids by adipocytes (page 3974, col. 2 and Fig. 8). The authors conclude that the antidiabetic effects of troglitazone may be due to improved fatty acid uptake by adipocytes (page 3976, col. 2). Similarly, Martin *et al.* (J. Biol. Chem. 272:28210-28217 (1997); copy enclosed as Exhibit 2) found that the antidiabetic thiazolidinedione BRL 49653 "resulted in a significant induction of adipose tissue FATP (7-fold) ... mRNA levels" (page 28213, col. 2). The induction of FATP mRNA also resulted in increased FFA uptake into adipocytes (page 28214, col. 2). The authors conclude that "thiazolidinedione antidiabetic agents seem to favor adipocyte-specific FA uptake relative to muscle, perhaps underlying in part the beneficial effects of these agents on insulin-mediated glucose dispersal" (Abstract). Thus the art at the time of filing demonstrated that agents which increased FFA uptake by adipocytes were useful in the treatment of diabetes.

The Examiner asserts that "there is still no indication that compounds in the art which increase uptake of FFA alone (not increasing glucose uptake) are effective for the treatment of any particular disease." (Page 17 of the instant Office Action).

As discussed above, circulating FFA levels regulate glucose uptake by adipocytes. Thus, even if the actual mechanistic effect of PRO1303 is only to directly increase FFA uptake by adipocyte cells, this will necessarily result in indirectly increasing glucose uptake by adipocytes. The effect of FFA levels on glucose uptake has been clearly demonstrated in the references previously cited by the Examiner. Furthermore, agents which are well known in the art as useful in the treatment of diabetes, such as the thiazolidenediones, have been shown to exert their effects, at least in part, through the increase of FFA uptake by adipocytes. Accordingly, an agent which increases FFA uptake by adipocytes has the same utility in the treatment of disease as those recognized by the Examiner for agents which increase glucose uptake.

Based on the above arguments, Applicants have clearly demonstrated a credible, specific and substantial asserted utility for the PRO1303 polypeptide, for example in the treatment of disorders such as obesity, diabetes, and hyper- or hypo-insulinemia. Further, based on this utility

and the disclosure in the specification, one skilled in the art at the time the application was filed would know how to use the claimed antibodies, for example in the purification of PRO1303 for use in therapeutic applications.

In view of the above, Applicants request the Examiner to reconsider and withdraw the rejection of Claims 28-32 under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph.

II. Claim Rejections Under 35 U.S.C. §102(e)

Claims 28-32 remain rejected under 35 U.S.C. §102(e) as allegedly being anticipated by Ni *et al.* (U.S. Patent No. 6,566,498). Ni *et al.* teach an isolated human secreted polypeptide consisting of SEQ ID NO:6, which has two regions of 100% identity to SEQ ID NO:194, one region of 62 amino acids at the N-terminus, and one of 93 amino acids at the C-terminus of SEQ ID NO:194. The Examiner asserts that the two proteins are likely splice variants of each other and thus are very likely to have many exterior-exposed epitope domains in common. The Examiner further asserts that "because antigenic epitopes can be as low as 7 amino acids and preferably between 15 and 30 amino acids" allegedly "many of the antibodies taught by the reference which are directed against the protein of SEQ ID NO:6 would strongly cross-react with and specifically bind to the polypeptide of SEQ ID NO:194." (Page 18 of the instant Office Action).

Applicants respectfully point out that, as discussed in the Response filed July 5, 2005, Claim 28, and consequently, those claims dependent from Claim 28, recites "an antibody that specifically binds to the polypeptide of SEQ ID NO:194." (Emphasis added). Therefore, Claim 28 and the claims dependent from Claim 28, carrying its recitations, clearly refer to an antibody that is able to bind to a specific epitope of the PRO1303 polypeptide *without* cross reacting with another epitope, including those found in the sequence disclosed in Ni *et al.* In view of this, the Examiner errs in assuming that the antibodies claimed in the present application would display significant binding to the polypeptide of Ni *et al.*, and thus overlap with the antibodies of Ni *et al.* As a result of the requirement of specific binding, the claims pending in this application do not encompass antibodies that specifically bind to epitopes found in the polypeptide of Ni *et al.*

Clearly there exist specific epitopes in the SEQ ID NO:194 protein that are not found in the Ni protein. Applicants note that, as shown in the sequence alignment provided by the Examiner in the previous Office Action mailed April 5, 2005, SEQ ID NO:194 contains a 93 amino acid region in the middle of the protein that is not present in SEQ ID NO:6 of Ni *et al.* Thus this 93 amino acid region of SEQ ID NO:194 contains numerous epitopes not found in the protein of Ni *et al.* Accordingly, one of ordinary skill in the art would readily understand what is meant by antibodies which specifically bind to SEQ ID NO:194 (and not, for example, to the polypeptide of Ni *et al.*). Such antibodies would clearly include, for example, antibodies that bind to epitopes found within the central 93 amino acid region of SEQ ID NO:194. One of skill in the art would understand that specific epitopes of the PRO1303 polypeptide may also include residues from the overlapping regions, as part of a non-linear epitope.

One of ordinary skill in the art would further understand how to make and use such antibodies. The specification provides methods to determine whether an antibody specifically binds to epitopes possessed by SEQ ID NO:194. Routine methods of determining antibody binding specificities, including immunoprecipitation, or competitive binding assays such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA), are disclosed in the specification at, for example, page 373, lines 32-35. Methods of determining the binding affinities of antibodies using Scatchard analysis are disclosed at page 373, lines 35-36. In addition, a method of using competitive binding assays to determine if a peptide shares an antigenic determinant for a particular antibody with a PRO polypeptide is disclosed in the specification at page 488, lines 25-29.

The Examiner asserts that "even just one antibody taught by the reference which specifically binds to the Ni *et al.* protein and specifically binds to the polypeptide of SEQ ID NO:194 would anticipate the claimed invention." (Page 19 of the instant Office Action). Applicants respectfully submit that this statement is incorrect, since antibodies that <u>specifically</u> bind to SEQ ID NO:194 would not include antibodies that bind equally well to the protein of Ni *et al.*

The Examiner further asserts that "Applicant's definition of 'specific binding' is too restrictive and does not interpret the phrase as broadly as is reasonable." (Page 20 of the instant Office Action). The Examiner asserts that 'specific binding' "is not defined relative to whether or not the antibody specifically binds to the same epitope present in a different protein, especially since it is unclear what would constitute a different protein in this context." (Page 20 of the instant Office Action).

Applicants respectfully submit that terms must be interpreted in a manner reasonably consistent with what is known in the art. Given that, as the Examiner mentions, antigenic epitopes comprise at least 7 amino acids, one of ordinary skill in the art would not expect that a protein having a single amino acid difference from SEQ ID NO:194, to take the Examiner's example, could be distinguished antigenically. On the other hand, a splice variant having a 93 amino acid region not found in the most closely related protein could certainly be distinguished from that protein. As the Examiner explains, it is understood in the art that a 93 amino acid region is large enough to comprise an independent folding domain, which may well have an additional function not present in the shorter variant. Because it is well known in the art that splice variants may have different functions and expression patterns, the skilled artisan would want to be able to distinguish between such variants. The skilled artisan would further understand that it would be simple to make antibodies that would specifically bind only the longer variant, by making antibodies to the unique splice region of the longer protein. Thus there is nothing unreasonable or untenable in the idea of generating antibodies that 'specifically bind' to proteins having entire domains not found in a related variant.

Accordingly, Applicants respectfully submit that Ni *et al.* is not prior art under 102(e) and request the Examiner to reconsider and withdraw the present rejection..

CONCLUSION

All claims pending in the present application are believed to be in *prima facie* condition for allowance, and an early action to that effect is respectfully solicited.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. **08-1641** (Attorney's Docket No. **39780-2830 P1C15**). Please direct any calls in connection with this application to the undersigned at the number provided below.

Respectfully submitted,

Date: November 29, 2005

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Identification of a Functional Peroxisome Proliferator-responsive Element in the Murine Fatty Acid Transport Protein Gene*

(Received for publication, September 9, 1998)

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Fatty acid transport protein (FATP), a plasma membrane protein implicated in controlling adipocyte transmembrane fatty acid flux, is up-regulated as a consequence of adipocyte differentiation and down-regulated by insulin. Based upon the sequence of the FATP gene upstream region (Hui, T. Y., Frohnert, B. I., Smith, A. J., Schaffer, J. A., and Bernlohr, D. A. (1998) *J. Biol. Chem.* 273, 27420–27429) a putative peroxisome proliferator-activated receptor response element (PPRE) is present from −458 to −474. To determine whether the FATP PPRE was functional, and responded to lipid activators, transient transfection of FATP-luciferase reporter constructs into CV-1 and 3T3-L1 cells was carried out. In CV-1 cells, FATP-luciferase activity was up-regulated 4- and 5.5-fold, respectively, by PPARα and PPARγ in the presence of their respective activators in a PPRE-dependent mechanism. PPARδ, however, was unable to mediate transcriptional activation under any condition. In 3T3-L1 cells, the PPRE conferred a small but significant increase in expression in preadipocytes, as well as a more robust up-regulation of FATP expression in adipocytes. Furthermore, the PPRE conferred the ability for luciferase expression to be up-regulated by activators of both PPARγ and retinoid X receptor α (RXRα) in a synergistic manner. PPARα and PPARδ activators did not up-regulate FATP expression in 3T3-L1 adipocytes, however, suggesting that these two subtypes do not play a significant role in differentiation-dependent activation in fat cells. Electromobility shift assays showed that all three PPAR subtypes were able to bind specifically to the PPRE as heterodimers with RXRα. Nuclear extracts from 3T3-L1 adipocytes also showed a specific gel-shift complex with the FATP PPRE. To correlate the expression of FATP to its physiological function, treatment of 3T3-L1 adipocytes with PPARγ and RXRα activators resulted in an increased uptake of oleate. Moreover, linoleic acid, a physiological ligand, up-regulated FATP expression 2-fold in a PPRE-dependent manner. These results demonstrate that the FATP gene possesses a functional PPRE and is up-regulated by activators of PPARα and PPARγ, thereby linking the activity of the protein to the expression of its gene. Moreover, these results have implications for the mechanism by which certain PPARγ activators such as the antidiabetic thiazolidinedione drugs affect adipose lipid metabolism.

Obesity, defined as an excessive accumulation of body fat, has become an increasingly common health concern in industrialized societies. Excessive adiposity has been linked to the pathogenesis of many diseases, including type 2 diabetes mellitus, coronary artery disease, and hypertension. The increased awareness of the detrimental effects of obesity contributes to the search for a greater understanding of the molecular mechanisms controlling the accumulation of adipose tissue and its metabolism.

A central issue in the function of fat tissue is the method by which adipocytes take up and release fatty acids. This process has been the source of considerable debate (1–3). Because free fatty acids are hydrophobic, they freely crossed membranes by passive diffusion. However, studies in adipocytes, hepatocytes, jejunal enterocytes, skeletal muscle, and heart myocytes support a saturable, protein-mediated mechanism for fatty acid transport (4–8). Thus far, five putative mammalian fatty acid transporters have been identified: fatty acid-binding protein (plasma membrane) (9), 56-kDa renal fatty acid-binding protein (10), caveolin (11), fatty acid translocase (12), and fatty acid transport protein (FATP)[1] (13). FATP is an integral plasma membrane protein with four to six predicted membrane-spanning regions with the highest levels found in skeletal muscle, heart, and fat with lower levels in brain, kidney, lung, and liver. Although FATP mRNA is present at low levels in 3T3-L1 preadipocytes, it is up-regulated 5–7-fold as a consequence of adipose conversion (13, 14). This increase is consistent with the increase in oleic acid uptake shown during preadipocyte differentiation (15).

The differentiation of adipose precursor cells into adipocytes has been shown to be mediated by three groups of transcription factors: peroxisome proliferator-activated receptor γ (PPARγ 1 and 2) the CCAATT enhancer-binding proteins (C/EBP), and the sterol-response element binding proteins (SREBP or ADD1) (16). The importance of PPARs for the development and maintenance of the adipocyte phenotype can be more directly shown by the existence of peroxisome proliferator response elements (PPREs) in the promoters of several genes whose protein products are critical for lipid metabolism and the development of the adipocyte phenotype such as lipoprotein lipase, phosphoenolpyruvate carboxykinase, acyl-CoA synthetase, malic enzyme, and adipocyte lipid-binding protein (ALBP or aP2) (16–21).

PPARs constitute a subfamily of the steroid hormone receptor superfamily. PPARα is predominantly expressed in liver, heart, kidney, and adipose tissue, whereas PPARδ (also known as NUC1 or FAAR) shows a similar expression, with the ex-

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[1] The abbreviations used are: FATP, fatty acid transport protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PPRE, peroxisome proliferator response element; PPAR, peroxisome proliferator-activated receptor; Me_2SO, dimethyl sulfoxide; BSA, bovine serum albumin; RXR, retinoid X receptor.

ception of low levels in liver (22–25). Two isoforms of mouse PPARγ have been cloned, γ1 (22, 24, 26) and γ2 (19), which are transcribed from the same gene and alternatively spliced (27, 28). PPARγ1 is expressed in liver, heart, and kidney, similar to PPARα, whereas PPARγ2 is primarily expressed in adipose tissue (29). The two PPARγ isoforms do not appear to differ significantly in ligand binding affinities, ability to bind DNA response elements, or ability to activate transcription. The critical difference identified, thus far, is the distribution of expression.

FATP expression has been shown to be up-regulated in mouse liver by PPARα activators and in white adipose tissues by activators of PPARγ (30, 31). This evidence, together with the differentiation-dependent regulation of FATP in 3T3-L1 adipocytes, led us to investigate the upstream region of the recently cloned FATP gene (32) for a possible PPRE and to examine the role of the various PPAR subtypes in FATP expression. In this report we detail the regulation of the FATP gene, the identification of a functional PPRE, and its up-regulation by PPARα and PPARγ agonists.

EXPERIMENTAL PROCEDURES

Materials—Troglitazone and Delta Selective C were gifts from Alan Saltiel (Parke-Davis) and David E. Moller (Merck). WY14643 was purchased from Cayman Chemical. Linoleic acid and 9-*cis*-retinoic acid were purchased from Biomol. Expression plasmids prPPARα, pSG5-FAAR (PPARδ), pBS-PPARγ2, and pRS-hRXRα were provided by Drs. Donald Jump, Paul A. Grimaldi, Bruce M. Spiegelman, David J. Mangelsdorf, and Ronald M. Evans, respectively.

Plasmid Constructs—Polymerase chain reaction was used to introduce *Nhe*I sites into the upstream sequence of FATP. Reporter constructs pNH11 (−971/+84), pNH13 (−556/+84), and pNH15 (−160/+84) were constructed by ligating varying lengths of FATP upstream sequence into the *Nhe*I and *Hin*dIII sites of the pGL3-Basic luciferase expression vector (Promega). Construct pNH13ΔP was generated by single-stranded mutagenesis of construct pNH13 using the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad, Hercules, CA) and the oligonucleotide: 5′-CTGGAACATCTCCTGAGTACTTCCTCCTCTCCC-3′. Sequence fidelity and the introduced mutation were verified by sequencing. The PPARγ2 expression construct, pAH215, was made by cutting pBS-PPARγ2 with *Kpn*I and *Xba*I and ligating into pCDNA3.1 (Promega). The expression construct for RXRα, pAH232, was subcloned from pRS-hRXRα into the *Eco*RI site of pCDNA3.1.

Cell Culture and Transfection—CV-1 cells were cultured in DMEM with 10% fetal bovine serum (FBS) until the day prior to transfection experiments, when the cells were plated in phenol red-free DMEM and 10% charcoal-treated FBS. CV-1 cells were transiently transfected using the calcium-phosphate method as described previously (33, 34). Briefly, each well of a 12-well culture plate was transfected with 1 μg of firefly (*Photinus pyralis*) luciferase reporter construct with or without 0.5 μg of expression plasmid for mouse PPARγ2, rat PPARα, or mouse PPARδ. 20 ng of pRL-SV40 (Promega), a vector containing a sea pansy (*Renilla reniformis*) luciferase gene driven by an SV40 promoter, was cotransfected into each well as a transfection control. After approximately 20 h, the calcium phosphate/DNA precipitate was removed by washing three times with phosphate-buffered saline. Cells were then refed and treated for 48 h with either the appropriate PPAR activator or its vehicle, dimethyl sulfoxide (Me_2SO).

3T3-L1 preadipocytes were grown to confluence and induced to differentiate to adipocytes as described previously(35). Briefly, preadipocytes were cultured in DMEM and 10% calf serum. Two days after reaching confluence, cells were differentiated by treatment with 174 nM insulin, 0.5 mM methylisobutylxanthine, and 0.25 μM dexamethasone in DMEM containing 10% FBS. Cells were treated for an additional 2 days with insulin and maintained in DMEM with 10% FBS until they are fully mature adipocytes at day 8.

Transient transfection of 3T3-L1 preadipocytes was performed by calcium phosphate precipitation as described above. Each well was transfected with 1 μg of firefly luciferase reporter construct and 10 ng of pRL-SV40 control vector. In the first set of transfections, cells were maintained in DMEM containing 10% calf serum until transfection mixture was washed off of cells. Cells were then treated with either linoleic acid or ethanol for 24 h in serum-free DMEM. For the second set of preadipocyte transfections, cells were maintained in DMEM with 10% calf serum for the duration of the experiments. After washing transfection mixture from cells, they were treated with either a PPAR activator or its vehicle (Me_2SO) for 48 h.

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-600 ATGAGGGGTC AAGAGCCCTA AACCTAAGCC TAAGAGCTTA AACTTAGCAC
-550 TCTATAAACT AGGAGAAGGG GGGGGGGGCG CGAAGAAAGA GGAAGGGAGT
-500 AGAGGGCACG AGCTGGGGAG AGGAGGAAGT GGGGCAAAGG GCACAGGAGA
-450 TGTTCCAGGA ACATCGAAAC AGGAGGAGGC AGTGAGCAAG TAGGAGGGCA
-400 GGCAGCAGGG CTGAATCCCA GCTGGGGGGG ACGGCTGCAG CATTCAGTGT
-350 CTTTACCTGT AAAACTGGGG TAGAGGGGGA GAGGGCTGGT CTAGAGGGTT
-300 CTTGTTGACC CCTGGCTGGC TGGGCCGAGA TCTTCATCAC CACCACCACC
-250 CCCATCCCAA CCACCTCTCT GAGACCAATG GCAGCTTCGG GTGGGGGTGG
-200 GGCCGCACTT GCCCAACCTT GGCCTTCGAG TGGCGCTGGA AACAGGCCCT
-150 GGCTGGCTTC TTCCCAAGGA GAACTGTGGG AAGGTCGGCC AAGCGGGACA
-100 GAAAACAAAC TAATCCCCAG GGTGGGCAGG GGGGCGTGGG AGGGTGGTTG
 -50 GGAGGGAGGG ACAGGGTAGA GGCACTCAGG GGCGCTGTGA GAAACCCCCA
 +1 GGACTCTTCA
```

FIG. 1. **Upstream sequence of the FATP gene.** The putative PPRE is *double underlined*.

3T3-L1 adipocytes grown in 12-well culture plates were transiently transfected as described previously (32) with 500 ng of firefly luciferase construct and 20 ng of pRL-SV40 control vector per well. Briefly, DNA in DMEM was complexed with LipofectAMINE (Life Technologies, Inc.) at the ratio of 1:8 (w/v) for 45 min before addition to the cells. After 4 h, an equal volume of 20% fetal bovine serum in DMEM was added. The transfection mixture was removed after 20 h. The cells were then refed with DMEM containing 10% FBS and treated with either PPAR activator or vehicle for 48 h.

All transiently transfected cells were washed two times with phosphate-buffered saline, lysed, and luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Firefly luciferase activities were normalized to sea pansy luciferase activities to adjust for transfection efficiency. Activities were also normalized to the promoterless reporter construct, pGL3-Basic.

In Vitro Transcription / Translation—cDNAs for rPPARα, mPPARγ2, mPPARδ, and hRXRα were transcribed and translated *in vitro* from the plasmids prPPARα, pAH215, pSG5-FAAR, and pAH232, respectively. The TNT Coupled Reticulocyte Lysate System (Promega) was used according to the manufacturer's instructions. The following expression plasmids were used prPPARα, pSG5-FAAR (PPARδ), pAH215, and pAH232. Translation products were verified by SDS-polyacrylamide gel electrophoresis.

Gel Electromobility Shift Assays—Nuclear extracts were prepared from day 9 3T3-L1 adipocytes essentially as described previously (36). To study the binding of nuclear hormone receptors to the putative PPRE, a double-stranded oligonucleotide, PPREwt, spanning nucleotides −482 to −453 of the FATP upstream sequence was ^{32}P-labeled with polynucleotide kinase (Promega). A 15-μl reaction containing and 0.5 ng of PPRE probe and 9 μg of nuclear extract or 0.5–1 μl of *in vitro* translation reaction was incubated for 20 min at 25 °C and 15 min at 4 °C in a buffer containing 20 mM HEPES (pH 8), 60 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 2 μg poly(dI-dC). The DNA-protein complexes were resolved from the free probe by electrophoresis at 4 °C on a 4% polyacrylamide gel in 0.5 × TBE buffer (1 × TBE = 9 mM Tris, 90 mM boric acid, 20 mM EDTA) (pH 8). Double-stranded oligonucleotides composed of the following sequences were used for competition analysis: PPREwt, 5′-GATCTAGAGGAGGAAGTGGGGCAAAGGGCA-CAGGA-3′; PPREmut, 5′-GATCTAGAGGAGGAAGTGGGGCtAtcGGC-ACAGGA-3. PPRE sequence is underlined. Mutated bases are shown in lowercase letters.

Fatty Acid Uptake—3T3-L1 adipocytes between days 7 and 9 were treated for 4 days with either 20 μM troglitazone and 1 μM 9-*cis*-retinoic acid or their carrier, Me_2SO. The adipocytes were then assayed for uptake of oleate essentially as described previously (37). Briefly, cells were first preincubated for 2–4 h in serum-free DMEM and then for 10–30 min at 25 °C in Krebs-Ringer phosphate solution with 2 mM glucose. Oleate uptake was measured at 25 °C by incubating cells in a 100 μM, 1:1 oleate:BSA mixture, which contained trace [^3H]oleate (approximately 12,000 dpm/nmol). At various time points, uptake was terminated by aspirating the oleate:BSA mixture from

TABLE I
Comparison of identified PPRE sequences

Consensus sequence derived from listed PPREs. Uppercase letters denote most conserved base(s); lowercase letters indicate a less conserved base alternative. HMG, hydroxymethylglutaryl; PEPCK, phosphoenolpyruvate carboxykinase.

Gene	Species	Element	Sequence	Protein function	Ref.
HMG-CoA synthase	Rat	HMG	AACT GGGCCA A AGGTCT	Liver ketogenesis/sterol synthesis	48
Acyl-CoA synthase	Rat	ACS(CI)	TTTC AGGGCA T CAGTCA	Fatty acid activation	20
Acyl-CoA oxidase	Rat	ACOA	GACC AGGACA A AGGTCA	Peroxisomal β-oxidation	52, 53
		ACOB	AGCA AGGTAG A AGGTCA		
	Human	hACOX	TAGA AGGTCA C TGGTCA	Peroxisomal β-oxidation	47
Bifunctional enzyme	Rat	BIF	ATGT AGGTAA T AGTTCA	Peroxisomal β-oxidation	54
Malic enzyme	Rat	MEp	TTCT GGGTCA A AGTTGA	Fatty acid synthesis	21
Cytochrome P450 A1	Rat	CYP4A1	AACT AGGGTA A AGTTCA	ω-Oxidation	46
Cytochrome P450 A6	Rabbit	CYP4A6(Z)	AACT AGGGCA A AGTTGA	ω-Oxidation	55
PEPCK	Rat	PCK1	CCCA CGGCCA A AGGTCA	Glycerogenesis and gluconeogenesis	17
		PCK2	AACT GGGATA A AGGTCT		
ALBP/aP2	Mouse	ARE6	CTCT GGGTGA A ATGTGC	Fatty acid binding	19
		ARE7	TACT GGATCA G AGTTCA		
L-FABP	Rat	FABP	ATAT AGGCCA T AGGTCA	Fatty acid binding	56
Uncoupling protein 1	Mouse	URE1	AGTG TGGTCA A GGGTGA	Thermogenesis	50
Lipoprotein lipase	Rat	LPL	AAGA GGGGGA A AGGGCA	Triglyceride clearance	18
Apolipoprotein CIII	Human	APOCIIIB	GCGC TGGGCA A AGGTCA	Triglyceride clearance	57
Muscle-type carnitine Palmitoyltransferase	Human	MCPT I	ATGT AGGGAA A AGGTCA	Fatty acid transport	49
Fatty acid transport Protein		FATP	AAGT GGGGCA A AGGGCA	Fatty acid transport	
		Consensus	AACT AGGTCA A AGGTCA Tg g G		

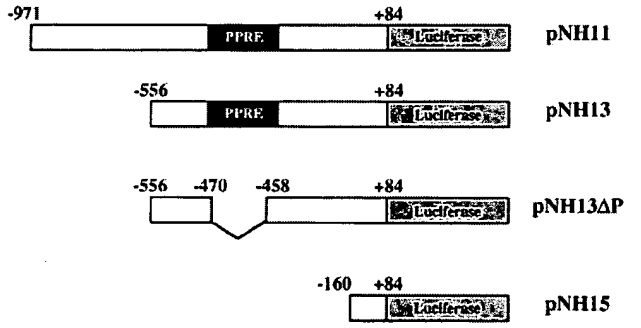

FIG. 2. **Construct map of the luciferase reporter constructs used in transfection assays.** Constructs contain various portions of FATP upstream sequence, as indicated. The putative PPRE is denoted by a *shaded box*.

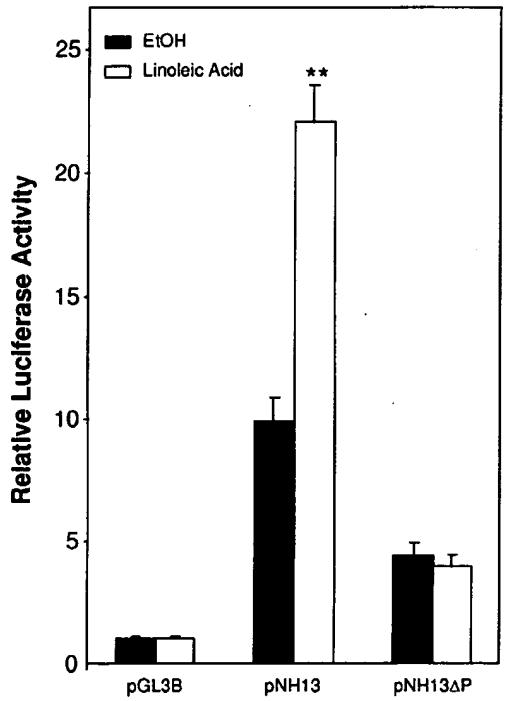

FIG. 3. **Activation of FATP expression in 3T3-L1 preadipocytes by linoleic acid.** 3T3-L1 preadipocytes were transfected with the reporter constructs pNH13 and pNH13ΔP, as well as the control construct, pGL3-Basic. The cells were then treated for 24 h in serum-free medium with either vehicle (EtOH) or 100 μM linoleic acid. *Asterisks* indicate statistical difference from activity of the control-treated construct (** = $p < 0.005$).

the cells and washing three times with ice-cold phosphate-buffered saline containing 0.1% albumin and 200 μM phloretin. Cell lysate was then quantitated for radioactivity using a Beckman 3801 liquid scintillation counter.

RESULTS

The Mouse Fatty Acid Transport Protein Gene Contains a Functional PPRE—Previous studies of FATP regulation in mice indicated that transcription was activated in both liver and white adipose tissue by treatment of mice with activators of PPARα or PPARγ, respectively (30, 31). Furthermore, FATP expression was observed to be up-regulated during adipose differentiation, a process known to be mediated in part by PPARγ (14). The upstream sequence of the recently cloned FATP gene (32) was therefore examined for a possible PPRE. A putative PPRE was identified, which is similar to the consensus sequence proposed by Palmer *et al.* (38) (Fig. 1, Table I).

In order to determine whether the putative PPRE identified in the FATP upstream sequence was able to mediate transcriptional activation, portions of the FATP 5′-flanking sequence were tested for their ability to mediate PPAR-activated transcription of a reporter gene. Four luciferase reporter constructs were made, each containing portions of the FATP 5′-flanking region linked to a promoterless firefly luciferase gene. Two of these plasmids, pNH11 and pNH13, contained the putative PPRE sequence, while the other two, pNH13ΔP and pNH15 did not (Fig. 2).

In the first set of experiments, two of these constructs, pNH13 and pNH13ΔP, as well as the promoterless pGL3-Basic control construct, were transfected into 3T3-L1 preadipocytes. Following transfection, the cells were maintained for 24 h in serum-free media and treated with 100 μM linoleic acid or its

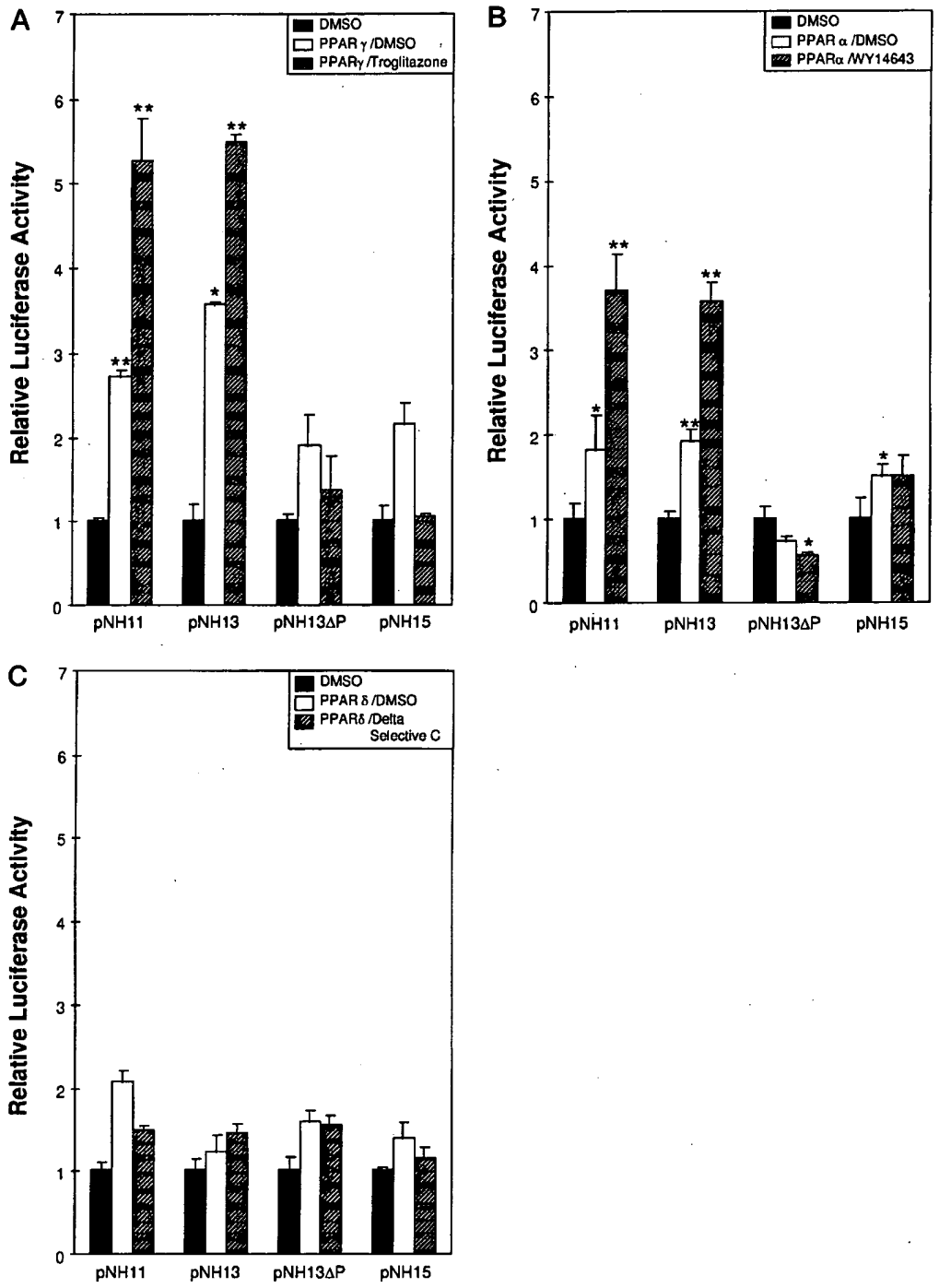

FIG. 4. **The putative PPRE confers selective responsiveness to PPAR-mediated activation.** Reporter constructs were cotransfected with or without an expression vector for PPAR γ (*A*), PPAR α (*B*), or PPAR δ (*C*) and treated for 48 h with activator or vehicle (Me_2SO (*DMSO*)). Activators were 20 μM Troglitazone, 10 μM WY14643, or 40 nM Delta Selective C, respectively. Normalized luciferase activities are shown as mean ± S.E. ($n = 4$) and are expressed as -fold induction relative to the activity in the absence of expression vectors and activators. *Asterisks* indicate statistical difference from activity of the reporter construct alone (* = $p < 0.05$, ** = $p < 0.005$).

carrier, ethanol. This experiment was performed in the absence of serum, since serum albumin binds fatty acids, leaving low levels of available fatty acid activator. Linoleic acid treatment activated transcription of the PPRE-containing construct approximately 2-fold over control-treated cells (Fig. 3), but did not affect transcription of the PPRE-deletion construct. Similar results were seen in transfection of 3T3-L1 adipocytes (data not shown). These experiments indicated that the putative PPRE identified in the FATP upstream sequence was indeed functional.

To further characterize the responsiveness of this PPRE to the various PPAR subtypes, all four reporter constructs were transfected into CV-1 cells and assayed for luciferase activity in the presence and absence of various PPARs and their activators. The activators used were troglitazone, a thiazolidinedione (PPARγ activator); WY14643, a fibrate drug (PPARα activator); and the PPARδ activator, Delta Selective C. As shown in Fig. 4*A*, cells transfected with PPARγ and treated with troglitazone demonstrated a 5.5-fold increase in transcription of the PPRE-containing reporter constructs. PPARγ alone was able to activate transcription approximately 3-fold. PPARα was also

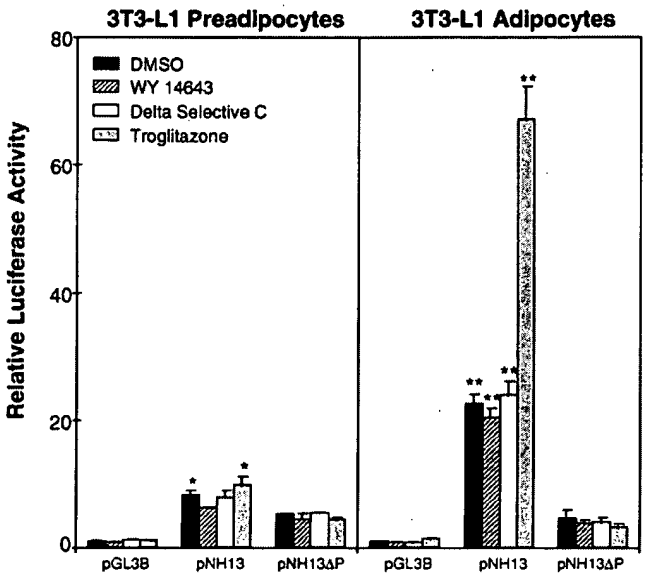

FIG. 5. **Activation of FATP expression by endogenous PPARs in preadipocytes and adipocytes.** 3T3-L1 preadipocytes and adipocytes were transfected with the reporter constructs pNH13 and pNH13ΔP as well as the control construct, pGL3-Basic. The cells were then treated with either vehicle or PPAR activators: 20 μM troglitazone, 10 μM WY14643, or 40 nM Delta Selective C. Luciferase activities were normalized to pGL3-Basic and are shown as mean ± S.E. (n = 4). *Asterisks* indicate statistical difference between pNH13 and pNH13ΔP expression (* = $p < 0.05$, ** = $p < 0.005$).

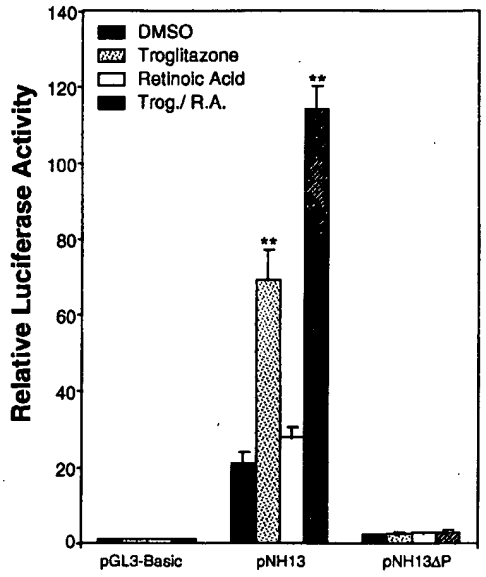

FIG. 6. **Synergistic activation of FATP expression in 3T3-L1 adipocytes by PPAR and RXRα activators.** 3T3-L1 preadipocytes and adipocytes were transfected with the reporter constructs pNH13 and pNH13ΔP as well as the control construct, pGL3-Basic. These cells were then treated with the PPARγ activator, troglitazone (20 μM), and the RXRα activator, 9-*cis*-retinoic acid (1 μM). Luciferase activities were normalized to pGL3-Basic and are shown as mean ± S.E. (n = 4). *Asterisks* indicate statistical difference from control-treated reporter construct (** = $p < 0.005$).

able to activate transcription in the PPRE-containing constructs, albeit to a lesser extent; transcription was increased 4- and 2-fold in the presence and absence of activator, respectively (Fig. 4*B*). Finally, the PPARδ subtype was unable to positively regulate transcription of any of the reporter constructs, regardless of activator treatment (Fig. 4*C*). Deletion of the PPRE rendered the promoter unresponsive to any PPAR or agonist combination.

PPRE Involved in Differentiation-dependent Regulation of FATP—In order to determine the role of the FATP PPRE in the process of its gene regulation during the process of adipose differentiation, the luciferase reporter constructs were introduced into both 3T3-L1 preadipocytes as well as mature adipocytes. These experiments relied upon endogenous PPARs to mediate transcriptional activation. Cells were also treated with activators of the various PPAR subtypes to determine whether transcription could be further increased. In preadipocytes, which contain low levels of PPARγ as well as PPARδ, the PPRE containing construct, pNH13, was expressed at about 1.6-fold the level of the PPRE-deletion construct, pNH13ΔP, when both were treated with Me_2SO. Treatment with troglitazone had a small, but not statistically significant, effect on pNH13 expression. In adipocytes, the PPRE-containing construct was expressed at levels 5-fold higher than the PPRE-deletion construct (Fig. 5). Furthermore, troglitazone treatment resulted in a further 3-fold increase in expression. Neither WY14643 nor Delta Selective C caused any significant change in luciferase expression in either preadipocytes or adipocytes.

Synergistic Activation by PPARγ and RXRα Activators—Issemann *et al.* (39) showed that the RXR ligand, 9-*cis*-retinoic acid enhances PPAR action. To determine whether RXRα activation affected PPARγ-dependent transactivation, 3T3-L1 adipocytes were treated with either troglitazone, 9-*cis*-retinoic acid, or both. Retinoic acid did not significantly affect transcription by itself; however, when added to cells in conjunction with troglitazone, it was able to produce an almost 2-fold increase in activity above that produced by troglitazone alone (Fig. 6). This result demonstrates the synergistic activation of the FATP gene in response to activation of both PPARγ and RXRα.

PPARs and RXRα Bind as Heterodimers to the FATP PPRE—In order to determine whether PPARs bind to the PPRE as heterodimers with RXRα, gel mobility shift assays were performed with a double-stranded oligonucleotide containing the FATP PPRE (Fig. 7*A*). The double-stranded probe, PPREwt, was end-labeled with ^{32}P and incubated with *in vitro* translated protein as well as 3T3-L1 nuclear extract. As shown in Fig. 7*B*, neither PPARs nor RXRα alone could bind to the PPRE; however, all three PPAR subtypes were able to bind as heterodimers with RXRα to the probe. Furthermore, nuclear proteins from 3T3-L1 adipocytes were able to form an *in vitro* complex with the PPRE (Fig. 7*C*). In order to test the specificity of the protein-DNA interactions, an excess of unlabeled oligonucleotide (PPREwt) was added to the reactions. The unlabeled oligonucleotide was able to compete for binding of all three PPAR-RXRα-DNA complexes, as well as the nuclear protein-DNA complex. The introduction of 3-base pair substitutions (Fig. 7*A*) produced an oligonucleotide, PPREmut, which was no longer able to significantly compete for protein binding.

PPARγ and RXRα Activators Cause an Increase in Oleate Uptake—Finally, in order to correlate PPAR-mediated transcriptional activation with putative *in vitro* protein function, fatty acid uptake was analyzed in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated for 4 days with either troglitazone and retinoic acid or their carrier Me_2SO. Cells were then incubated with [^{3}H]oleate:BSA mixture (1:1), washed, lysed at various time points, and assayed for radioactivity. As shown in Fig. 8, treatment with the activators of PPARγ and RXRα resulted in a significant increase in oleate uptake. This result is consistent with the up-regulation of FATP transcription by troglitazone and 9-*cis*-retinoic acid, as shown by the previously described transfection studies, and correlates well with the increase in FATP mRNA expression upon treatment of 3T3-L1 adipocytes

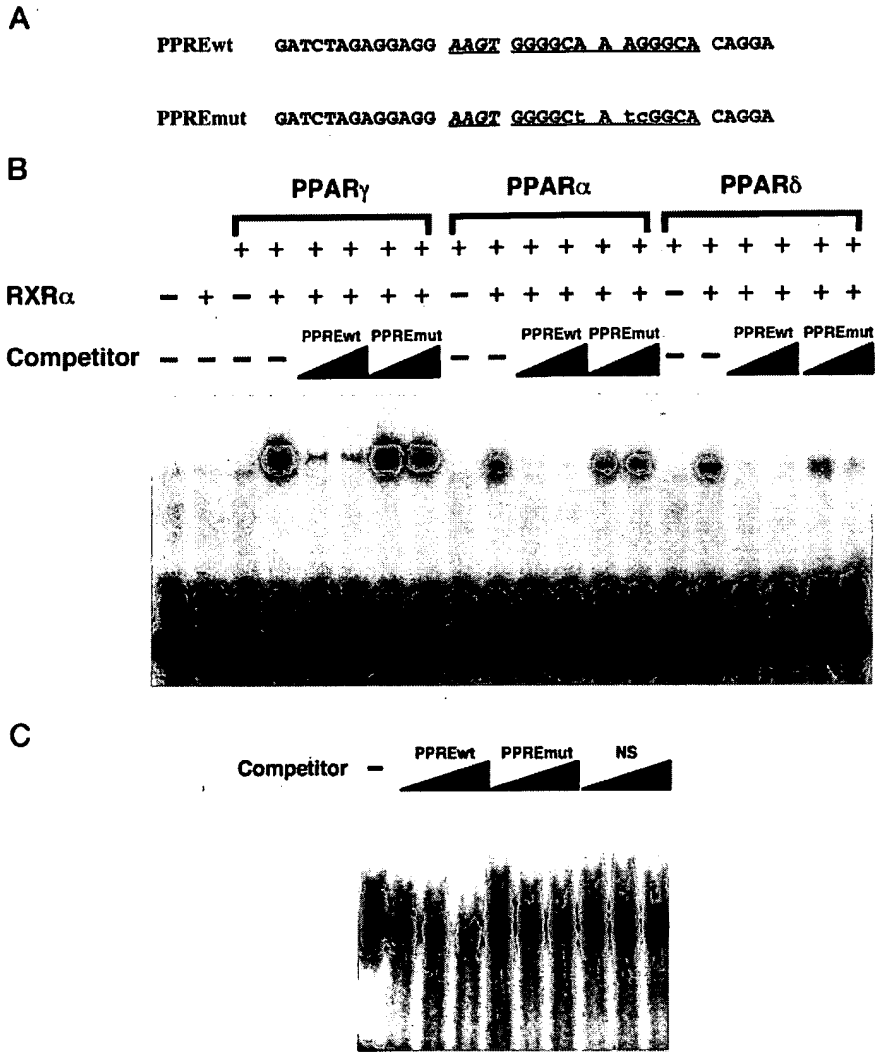

FIG. 7. **PPARs and RXRα bind as heterodimers to the FATP PPRE.** *A*, sequences of oligonucleotides used in gel-shift studies. PPRE sequence is *underlined*; mutated bases are in *lowercase*. *B*, the double-stranded probe, PPREwt, was end-labeled with ^{32}P and incubated with *in vitro* translated PPARs and RXRα. The competitors PPREwt and PPREmut were used in 20- and 100-fold molar excess. Protein-DNA complexes were analyzed by electrophoretic mobility shift assay. *C*, labeled PPREwt probe was incubated with 3T3-L1 adipocyte nuclear extracts with or without competitor. Competitors PPREwt, PPREmut, and NS (a nonspecific competitor oligonucleotide) were added in 20-, 50-, and 200-fold molar excess.

with the PPARγ agonist BRL49653, as shown by Martin *et al.* (30).

DISCUSSION

The critical role of PPARs in the regulation of lipid metabolism has become increasingly apparent. Many genes whose products take part in some aspect of fatty acid catabolism, synthesis, or trafficking have been shown to contain functional PPREs (Table I). FATP, which has been argued to play a role fatty acid uptake, is a likely candidate for regulation by this group of nuclear hormone receptors. Indeed, two previous studies have shown that treatment of various cell types with PPAR activators leads to an increase in FATP mRNA levels (30, 31). In this paper we have identified a sequence in the 5′ region of the murine FATP gene, which is very similar (16/17) to the consensus sequence for previously identified PPREs (see Table I).

By deletion analysis and mutation of this putative PPRE, we have demonstrated that this PPRE is necessary for the PPAR-mediated up-regulation of FATP expression. Transfection into 3T3-L1 preadipocytes showed that FATP transcription can indeed be activated by the naturally occurring compound, linoleic acid. This fatty acid has been shown previously to be able to activate transcription via both the α and γ PPAR subtypes (40–42). Further transfection experiments into CV-1 cells demonstrated that FATP transcription was activated by both PPARα and PPARγ. Both subtypes activate transcription upon treatment with synthetic activator; however, the receptors are also able to activate transcription in the absence of exogenous activator. This could be explained either by the presence of an endogenous activator, such as a fatty acid or its metabolite, or by a ligand-independent activity of these subtypes (43).

In addition to showing the functionality of the FATP PPRE, our experiments demonstrate a differential activation of gene expression by the various PPAR subtypes. Although both PPARα and PPARγ are able to activate transcription, our studies show that PPARγ mediates a greater response both in the presence and absence of a synthetic activator. PPARδ, in contrast, did not significantly activate transcription, either alone or upon treatment with activator. This difference has been demonstrated previously in other systems (44) and has been hypothesized to reflect the differing roles of the PPAR subtypes in the regulation of fatty acid metabolism. PPARα has been best characterized in the liver, where it up-regulates many genes involved in the catabolism of fatty acids. PPARγ is chiefly active in the adipose tissue, where it contributes to lipid accumulation and the development of the adipose phenotype. In contrast to the other two subtypes, the role of PPARδ in whole-body fatty acid metabolism has not been well defined.

We and others have reported the up-regulation of FATP during adipose conversion (13, 14). We hypothesized that this differentiation-dependent regulation of FATP is mediated by PPARγ and is dependent on the presence of the FATP PPRE. This was investigated in a further series of transfections, which

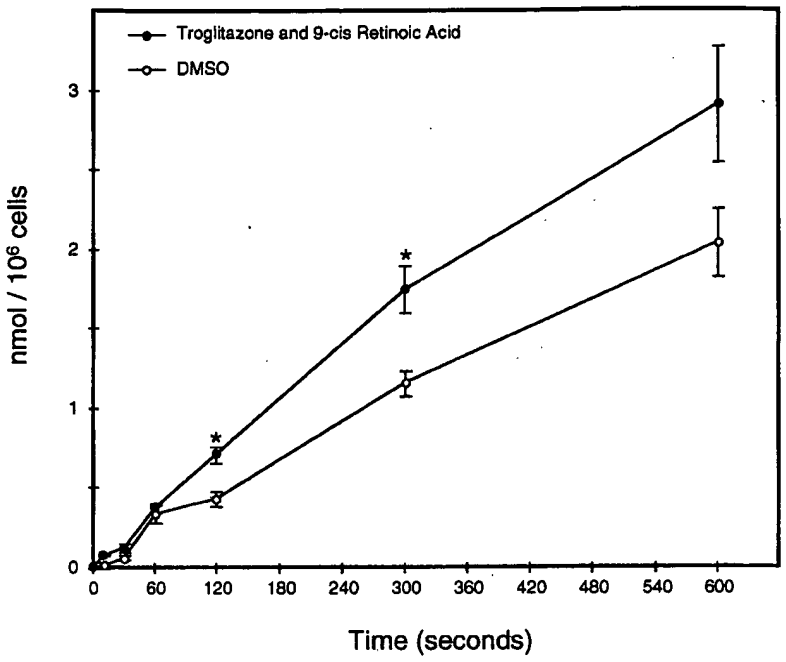

FIG. 8. **Oleate uptake by 3T3-L1 adipocytes treated with troglitazone and retinoic acid.** 3T3-L1 adipocytes were treated for 4 days with either 20 μM troglitazone and 1 μM 9-*cis*-retinoic acid or their carrier Me_2SO (*DMSO*). Cells were then incubated with 1:1 oleate:BSA mixture in Krebs-Ringer phosphate buffer which contained trace [^{3}H]oleate. Cells were washed and lysed at various time points and cell lysate was assayed for radioactivity. *Asterisks* indicate statistical difference between uptake of treated and nontreated cells (* = $p < 0.05$).

compared transcriptional activity in both 3T3-L1 preadipocytes and adipocytes, in both the presence and absence of synthetic activators. The PPRE conferred a 1.6-fold activation of FATP transcription in preadipocytes. At this point in the differentiation process, PPARγ is present in low levels relative to fully differentiated adipocytes. Exogenous activators of the three PPAR subtypes did not significantly increase transcription above that of untreated cells. In adipocytes, the PPRE conferred a 5-fold increase in FATP expression, and this activation was further stimulated by the PPARγ activator, troglitazone. Neither the activator of PPARα nor PPARδ was able to increase FATP expression over untreated cells. This can be explained by the lack of significant PPARα expression in adipocytes. PPARδ, while present in adipocytes, has been demonstrated in the previously described studies to be unable to activate FATP expression.

Several previous studies have shown a synergistic effect of the RXRα activators, 9-*cis*-retinoic acid on PPAR-activated expression (18, 19, 21, 45–48). This convergence of the PPAR and RXR signaling pathways was also demonstrated in transfection studies of FATP reporter constructs in 3T3-L1 adipocytes. Although the RXRα activator alone was unable to increase expression of FATP in 3T3-L1 adipocytes, it was able to enhance the troglitazone-mediated activation of expression.

The ability of the various PPAR subtypes to bind to the FATP PPRE *in vitro* was examined by electromobility shift assay. As has been shown in other systems, neither the PPARs nor RXRα were able to bind to the PPRE as homodimers (20, 21, 45–50); however, all three PPAR subtypes were able to bind as heterodimers. These protein-DNA complexes were sequence-specific, as shown by competition analysis, and were dependent upon the presence of an intact PPRE. Mutation of 3 base pairs of the PPRE abolished the formation of protein complexes on this element. It is interesting to note that PPARδ, while unable to activate transcription, was able to form a heterodimer complex with the PPRE. This indicates that binding of the receptor heterodimers to an element is not equivalent with transcriptional activity.

In order to correlate FATP regulation by PPARs with its putative function, oleate uptake was measured in cells that were treated with troglitazone and 9-*cis*-retinoic acid. Uptake was significantly increased in activator-treated cells when compared with control-treated cells. This leads to an interesting model for positive feedback regulation of FATP. Increased FATP expression has been shown to result in increased fatty acid uptake (13). Fatty acids, in turn, are activators of PPARγ and PPARα, which are able up-regulate the expression of FATP.

We have reported previously the regulation of FATP by insulin via an insulin-responsive element (PLE3) in the upstream region of the FATP gene (−1353 to −1347). The down-regulation of FATP by insulin, an anabolic hormone, seems counterintuitive, since fatty acid uptake would be expected to rise in response to insulin stimulation. It is important to note, however, that regulation of FATP at the transcriptional level is unlikely to be the result of the transient postprandial insulin peak, but rather a more chronic hyperinsulinemia, such as in type 2 diabetes mellitus. Furthermore, the majority of type 2 diabetics are obese, a condition associated with a down-regulation of PPARγ expression in adipose tissue (51). The combination of these two factors may contribute to the elevation in serum free fatty acid levels observed in type 2 diabetics. This leads to a possible mechanism for the antidiabetic effects of the drug, troglitazone; by reversing the effects of hyperinsulinemia and obesity on FATP regulation, troglitazone may enable adipose tissue improve fatty acid uptake. Further studies on the function of FATP and its regulation in the diabetic state may lead to insight into both normal and deranged fatty acid metabolism.

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Coordinate Regulation of the Expression of the Fatty Acid Transport Protein and Acyl-CoA Synthetase Genes by PPARα and PPARγ Activators*

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Intracellular fatty acid (FA) concentrations are in part determined by a regulated import/export system that is controlled by two key proteins, *i.e.* fatty acid transport protein (FATP) and acyl-CoA synthetase (ACS), which respectively facilitate the transport of FAs across the cell membrane and their esterification to prevent their efflux. The aim of this investigation was to analyze the expression pattern of FATP and ACS and to determine whether their expression was altered by agents that affect FA metabolism through the activation of peroxisome proliferator-activated receptors (PPAR) such as the fibrates and thiazolidinediones. FATP mRNA was ubiquitously expressed, with highest levels being detected in adipose tissue, heart, brain, and testis. Fibrate treatment, which is known to preferentially activate PPARα, induced FATP mRNA levels in rat liver and intestine and induced ACS mRNA levels in liver and kidney. The antidiabetic thiazolidinedione BRL 49653, which is a high-affinity ligand for the adipocyte-specific PPARγ form, caused a small induction of muscle but a robust induction of adipose tissue FATP mRNA levels. BRL 49653 did not affect liver FATP and had a tendency to decrease heart FATP mRNA levels. ACS mRNA levels in general showed a similar pattern after BRL 49653 as FATP except for the muscle where ACS mRNA was induced. This regulation of FATP and ACS expression by PPAR activators was shown to be at the transcriptional level and could also be reproduced *in vitro* in cell culture systems. In the hepatocyte cell lines AML-12 or Fa 32, fenofibric acid, but not BRL 49653, induced FATP and ACS mRNA levels, whereas in the 3T3-L1 preadipocyte cell line, the PPARγ ligand induced FATP and ACS mRNA levels quicker than fenofibric acid. Inducibility of ACS and FATP mRNA by PPARα or γ activators correlated with the tissue-specific distribution of the respective PPARs and was furthermore associated with a concomitant increase in FA uptake. Most interestingly, thiazolidinedione antidiabetic agents seem to favor adipocyte-specific FA uptake relative to muscle, perhaps underlying in part the beneficial effects of these agents on insulin-mediated glucose disposal.

Transmembrane transport of FAs[1] is still poorly understood despite intense investigation. Uncharged molecules and weak acids such as fatty acids can cross membranes rapidly thanks to their lipid solubility. The rate of movement is controlled by mass action and can be enhanced by proteins such as fatty acid-binding protein that act as a cytoplasmic "sink." Recently, however, several studies provided evidence that in addition to these nonfacilitated systems, facilitated transport also contributes to FA transport (1). Several proteins were hypothesized to be acting as FA transporters. Among these, three deserve further attention. First, plasma membrane fatty acid-binding protein, a protein related to the mitochondrial isoform of aspartate aminotransferase, has been suggested to increase FA uptake in cells (2). Since this protein has not yet been cloned, it is difficult to determine its exact role in FA transport processes. The second protein, fatty acid translocase is an 88-kDa membrane protein that has been cloned in mouse and is homologous to the human CD36 cell surface antigen (3). Although CD36 has been shown to bind FAs and might be involved in signal transduction after binding of a specific ligand (long chain fatty acids), it is until now not clear whether it is a transport protein. The only candidate for a long chain FA transporter for which functionality has been directly demonstrated is the fatty acid transport protein (FATP) (4). FATP is a 63-kDa plasma membrane protein with six predicted membrane-spanning domains that has been cloned using a functional expression cloning technique. It increased oleic acid uptake in FATP-transfected 3T3-L1 cells by >3-fold. Interestingly FATP is suggested to act in concert with acyl-CoA synthetase (ACS), an enzyme that prevents efflux of the incorporated fatty acids by their conversion into acyl-CoA derivatives and hence rendering the FA uptake process unidirectional. Furthermore, FATP shows a limited region of homology at the protein level (11 amino acids), with ACS leading to the hypothesis that this common region might reflect a common function, such as a binding site (4). These 11 amino acids residues have also been found to be conserved in the rat and the yeast FATP homologues (5).

Several aspects of intracellular lipid and FA metabolism in cells are subjected to transcriptional control by the peroxisome proliferator-activated receptor (PPAR) family. PPARs are members of the superfamily of nuclear hormone receptors that function as ligand-dependent transcription factors. Three receptor subtypes of PPAR termed α, δ (or β), and γ, have been identified (6–15). These receptors heterodimerize with the retinoid X receptor and alter the transcription of target genes after binding to peroxisome proliferator response elements (PPREs), which consist of a hexameric nucleotide direct repeat of the recognition motif (TGACCT) spaced by 1 nucleotide

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[1] The abbreviations used are: FA, fatty acid; FATP, FA transport protein; ACS, acyl-CoA synthetase; PPAR, peroxisome proliferator-activated receptor; BSA, bovine serum albumin; LPL, lipoprotein lipase.

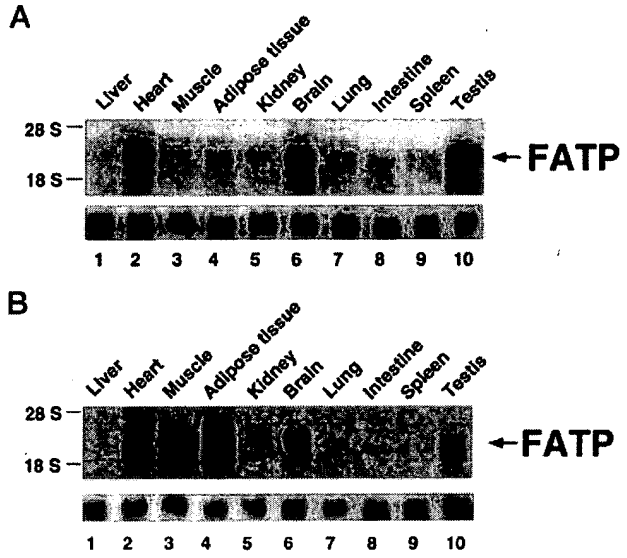

FIG. 1. **Tissue distribution of FATP mRNA expression in mouse (*A*) and rat (*B*).** Twenty μg of total RNA from the respective tissues was analyzed by Northern blot hybridization for FATP and 36B4 mRNA expression. RNA extraction and analysis was performed as described under "Experimental Procedures."

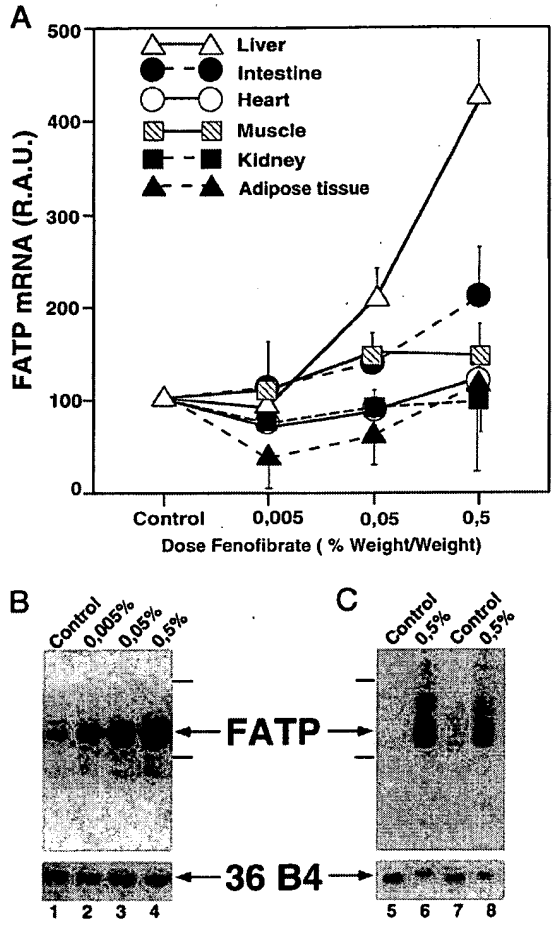

FIG. 2. **Effect of fenofibrate on FATP mRNA levels.** *A*, graph showing the effects fenofibrate mixed with food in the indicated concentrations on FATP mRNA levels. RNA extraction and analysis was performed as described under "Experimental Procedures." *R.A.U.*, relative absorbance units. *B*, effect of increasing amounts of fenofibrate on liver FATP expression in rat. Twenty μg of total liver RNA was analyzed by Northern blot hybridization for FATP and 36B4 mRNA expression as indicated under "Experimental Procedures." *C*, effect of fenofibrate on liver FATP expression in mouse. Twenty μg of total liver RNA tissues was analyzed by Northern blot hybridization for FATP and 36B4 mRNA expression as indicated under "Experimental Procedures."

(DR-1). Several genes with a crucial role in FA metabolism have been shown to contain a peroxisome proliferator response element in their upstream regulatory sequences (reviewed in Refs. 16 and 17). Interestingly, the transcriptional activity of the PPAR subtypes is enhanced by a multitude of chemical compounds including fatty acids, thiazolidinedione antidiabetic agents, prostaglandins, peroxisome proliferators, and fibrate hypolipidemic drugs. In addition to activating PPARs, some of these compounds have been shown to be direct ligands for them. PPARγ directly binds antidiabetic thiazolidinediones (18, 19) and prostaglandin derivatives (18, 20) but not the other activators, whereas PPARα binds leukotriene B4 and the powerful peroxisome proliferator Wy 14643 (21).

In view of the convergence of FA import and PPARs in lipid and energy metabolism, we investigated the effects of two distinct chemical classes of PPAR activators, *i.e.* fibrates (PPARα-specific) and the antidiabetic thiazolidinediones (PPARγ-specific) on tissue-specific *FATP* gene expression. Fibrate treatment induced FATP and ACS expression strongest in liver, whereas BRL 49653, the high affinity ligand for PPARγ, had no effect on liver but induced adipocyte FATP and ACS expression in adipose tissue. The induction of FATP and ACS by PPAR activators was at the level of transcription and was associated with concomitant changes in cellular FA uptake. Interestingly, the stronger effects of BRL 49653 on fatty acid import in adipose tissue relative to the muscle might limit FA uptake and oxidation in the muscle, an effect associated with an improvement in muscle glucose disposal.

EXPERIMENTAL PROCEDURES

Materials—BRL 49653 and fenofibric acid were kind gifts of Dr. De Chaffoy de Courcelles (Janssen Research Foundation, Beerse, Belgium) and Dr. Alan Edgar (Laboratoires Fournier, Daix, France), respectively.

Animal Studies—Animal studies were carried out in compliance with French and European union specifications regarding the use of laboratory animals. Male Wistar rats (90 days old) were treated for 7 days with fenofibrate (Laboratoires Fournier) mixed at the indicated concentrations (by mass) in standard rat chow. The food intake of the rats was recorded every day throughout the treatment period. None of the treatments caused major changes in the amount of food consumed by the animals. Since each rat consumed approximately 20 gm of chow/day, doses of 0.5, 0.05, and 0.005% (by mass mixed in rat chow) correspond to 320, 32, and 3 mg/kg of body weight/day. Adult (95 day) Sprague-Dawley rats were group-housed and accustomed to a 12:12 h day:night ratio illumination cycle (light from 8 am to 8 pm). Rats were divided in groups of a minimum of three animals each and were treated for either 7 or 14 days. The first group received BRL 49653 (5 mg/kg/day) by gavage. The second group of animals received 0.5% w/w of fenofibrate ($\pm$ 0.5 g/kg/day) mixed with their food, whereas the third group of animals served as controls and received 10% carboxymethylcellulose (vehicle for gavage) by gavage. In a separate experiment, adult C57Bl6 male mice were either fed during 14 days with a control chow (n = 3) or the same chow containing 0.5% w/w of fenofibrate. At the end of the treatment period, all animals were weighed and sacrificed by exsanguination under ether anesthesia between 8 and 10 a.m. Epididymal adipose tissue (in rats) and liver (in rats and mice) was removed, weighed, rinsed with 0.9% NaCl, and frozen in liquid nitrogen until RNA preparation.

Cell Culture—The mouse hepatoma and preadipocyte cell lines Fa 32 (rat), ob 1771 (mouse) (22), and 3T3-L1 (mouse; ATCC) were maintained in Dulbecco's modified Eagle's minimal essential medium and supplemented with 10% delipidated and charcoal-treated fetal calf serum, L-glutamine, and antibiotics unless stated otherwise. AML-12 mouse hepatocytes (23) were maintained in Dulbecco's modified Eagle's minimal essential medium/Ham's F-12 supplemented with 10% delipidated and charcoal-treated fetal calf serum, insulin, transferrin, and selenium (Collaborative Research), dexamethasone (0.1 μM), and gentamycin (50 μg/ml). Fenofibric acid and BRL 49653 (both in Me_2SO) were added to the medium at the appropriate concentrations and times

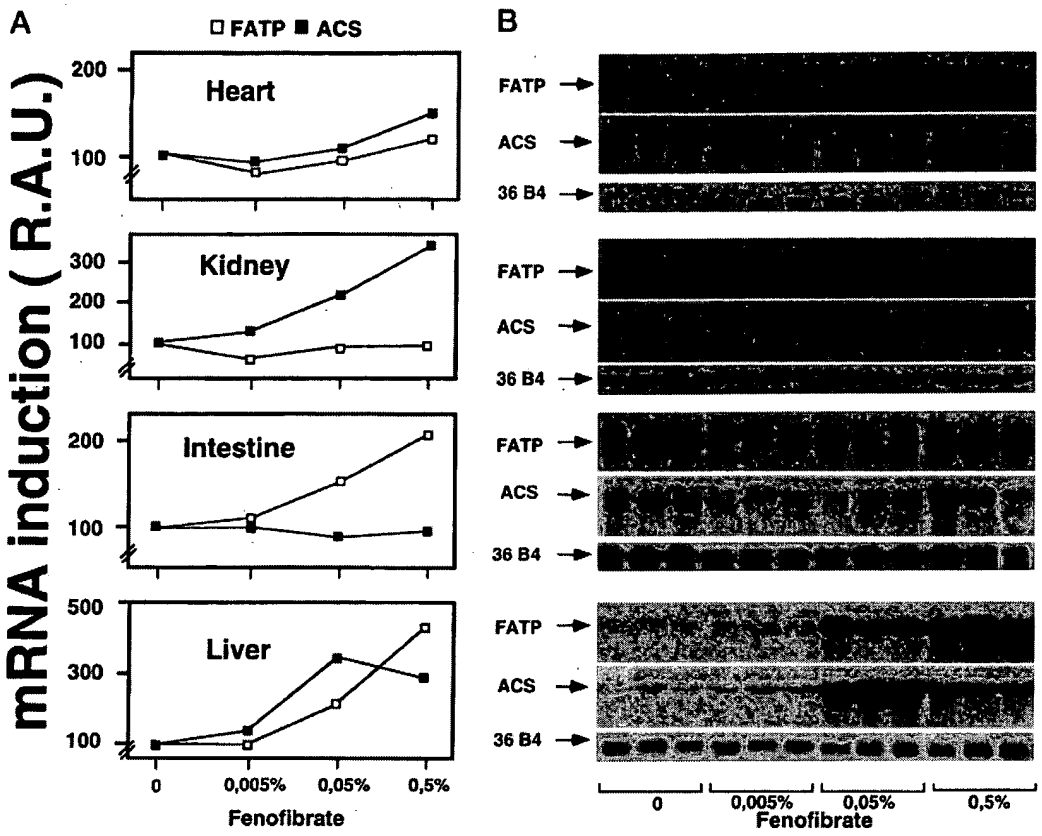

FIG. 3. **FATP and ACS mRNA are in some tissues coinduced by fenofibrate.** *A*, graphs representing the effects of three different concentrations of fenofibrate (0.005, 0.05, and 0.5% (w/w) during 14 days) mixed with food on FATP (*open squares*) or ACS (*filled squares*) mRNA levels. The results represent the mean of three independent samples. *B*, Northern blot showing the regulation of FATP, ACS, and 36B4 mRNA levels by fenofibrate administration. Animal treatment and preparation and analysis of RNA is described under "Experimental Procedures." *R.A.U.*, relative absorbance units.

indicated. Control cells received vehicle only.

3T3-L1 cells were differentiated by a treatment of 2 days with dexamethasone (0.1 μM), isobutylmethylxanthine (0.25 mM), and insulin (0.4 μM); the cells were then maintained for an additional 8 days with insulin until complete differentiation.

Preparation of Albumin-bound Fatty Acids—Radiolabeled ^{14}[C]oleate fatty acid was mixed in water at 40 °C, albumin (BSA; fraction V, fatty acid-free, Sigma) was then added from a concentrated stock (20 g/100 ml) to give a final molar ratio of 1/1 by gentle mixing. 2 × Hanks' solution was added to obtain a 1 × final solution. Incubation was carried out at 37 °C for 45 min.

Fatty Acid Uptake Assay—The measurement of uptake of 1-^{14}C-labeled oleate (about 50 mCi/mmol, NEN Life Science Products) was carried out in 24- or 6-well plates with 10^6 cells/ml of medium. Before treatment, the cells were washed with 1 × Hanks' solution. BRL 49653 (100–250 nM) and fenofibric acid (100–250 μM) were added in fresh Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum. After 48 h of treatment, cells were washed with Hanks' solution and incubated for 1 additional h in serum-free, glucose-free medium. Cells were then washed once at 37 °C and twice at 23 °C with 1 × Hanks' solution containing BSA. Hanks' solution without BSA was then added before the assay. A volume corresponding to 1 μCi of [^{14}C]oleate albumin-bound solution was added in each well, and cells were incubated for 1 min at room temperature. The incubation was stopped after 1 min with 3 washes of ice-cold 1 × Hanks' solution without BSA. A complementary experiment has been performed to verify whether aspecific cell surface binding of [^{14}C]oleate could interfere with the assay. For this second assay, the cells were washed under more stringent conditions in 1 × Hanks' solution containing 2% BSA. Cells were then lysed in 400 μl of 0.1% SDS solution. The lysate was counted for 5 min with 4 ml of scintillation solution. The assay was performed on triplicate points.

RNA Analysis—RNA preparation, Northern blot hybridizations, and quantification of total cellular RNA were performed as described previously (24). A mouse FATP cDNA probe was obtained after cloning a reverse transcription-polymerase chain reaction fragment from mouse adipose tissue RNA using the primers 382 (ATGCGGGCTCCTGGAGCAGGACAGCC) and 399 (CTGCGTGTCAGGCAGGATGCTCTCAGGCCC) into pBS-KS. The insert was sequenced and found to be identical to the reported mouse FATP sequence. The rat ACS probe corresponds to the *Eco*RV restriction fragment of the rat ACS cDNA. The human acidic ribosomal phosphoprotein 36B4 (25) was used as a control probe.

RESULTS

FATP mRNA Is Ubiquitously Expressed—To determine whether FATP expression was ubiquitous or restricted to certain tissues, we hybridized both a mouse (Fig. 1*A*) and a rat (Fig. 1*B*) multiple tissue Northern blot with a radiolabeled FATP probe. In both rat and mouse, adipose tissue, heart, brain, and testis showed the highest level of expression. Intestine and muscle show intermediate levels of expression, and low levels are expressed in the liver, kidney, lung, and spleen.

Fenofibrate, a PPARα Activator, Induces FATP mRNA in Vivo—In addition to being building blocks and energy substrates, fatty acids are also important signaling molecules. Besides being activated by peroxisome proliferators and certain thiazolidinediones, the transcriptional activity of PPARs can be activated by fatty acids (reviewed in Refs. 16 and 17). Therefore, we were interested in analyzing whether activation of these PPARs would affect FA uptake in general and FATP expression in particular. To address this issue we first assessed the effect of fibrates, potent PPARα activators, on the expression of the *fatp* gene in rats. Rats were hence treated with different doses of fenofibrate (14 days treatment at the doses 0.005, 0.05, and 0.5% by mass) mixed in food. Next, RNA was isolated from various organs and analyzed by Northern blot hybridization. In liver, FATP mRNA levels increased gradually

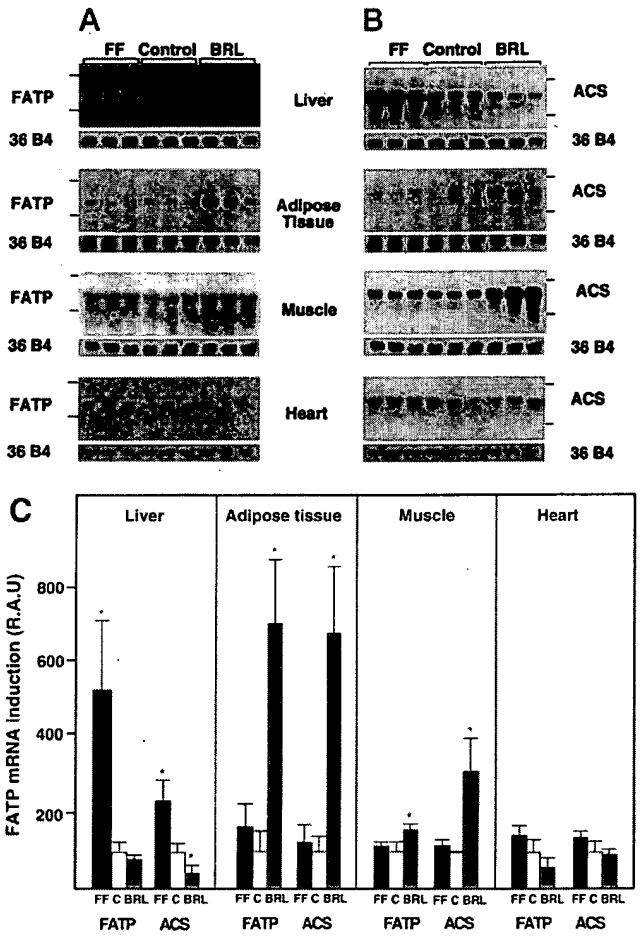

FIG. 4. **Tissue-selective induction of FATP (*A*) and ACS (*B*) mRNA in rat liver, adipose tissue, skeletal muscle, and heart by fenofibrate and BRL 49653, respectively.** *A*, expression of FATP mRNA in liver, epididymal adipose tissue, skeletal muscle, and heart of animals treated with fenofibrate (*FF*, 0.5% (w/w) during 7 days, approximately 0.5 g/kg/day) or BRL 49653 (5 mg/kg/day during 7 days). The blots were stripped and rehybridized with the human acidic ribosomal phosphoprotein 36B4 control cDNA. *B*, expression of ACS mRNA in liver, epididymal adipose tissue, skeletal muscle, and heart of animals treated with fenofibrate (*FF*, 0.5% (w/w) during 7 days, approximately 0.5 g/kg/day) or BRL 49653 (5 mg/kg/day during 7 days). The blots were stripped and rehybridized with the human acidic ribosomal phosphoprotein 36B4 control cDNA. Animal treatment and preparation and analysis of RNA is described under "Experimental Procedures." *C*, bar graph summarizing the regulation of ACS and FATP mRNA in liver, epididymal adipose tissue, skeletal muscle, and heart of animals treated with or without BRL 49653. *R.A.U.*, relative absorbance units. Values statistically significant from controls (Mann-Whitney; $p < 0.05$) are indicated by an *asterisk*.

starting from 0.05% fenofibrate and reached a maximal 4.2-fold induction at the highest dose of 0.5% fenofibrate (Fig. 2, *A* and *B*). A representative Northern blot showing the induction of FATP mRNA in the liver is depicted in Fig. 2*B*. Next, the response of FATP mRNA levels to fibrates was studied in intestine, skeletal muscle, heart, and kidney. Only intestinal FATP mRNA expression was slightly induced (2-fold) by the highest dose of fibrate treatment, whereas muscle, kidney, and heart FATP mRNA expression remained unchanged. Also in mice, administration of fenofibrate (0.5%) induced FATP mRNA levels in liver (15-fold) (Fig. 2*C*).

Parallels between the Fibrate Effects on FATP and ACS—Several proteins are hypothesized to enhance fatty acid uptake into cells. In contrast to FATP, which acts as an FA transport protein, ACS prevents the efflux from the imported FAs by converting them into acyl-CoA derivatives, which can subsequently be used in both anabolic and catabolic pathways. Therefore, we next analyzed whether there was a parallel between the induction of FATP and ACS after fibrate treatment (Fig. 3). Fenofibrate induced liver and kidney ACS mRNA expression, whereas no change in ACS expression was observed in heart and intestine. Therefore both FATP and ACS mRNA levels seem to be coordinately regulated in liver and heart, since fibrates affect both parameters in a similar fashion. The regulation of ACS and FATP in the kidney and intestine seems to be divergent, since in these tissues only one of the respective mRNAs is regulated by fibrate treatment.

PPARγ Activators Induce FATP mRNA in Adipose Tissue—In addition to the well established effects of peroxisome proliferators such as the different fibrates on PPARα activity, we next tested the effects of the PPARγ-selective ligand BRL 49653 on FATP and ACS expression in various rat tissues after administration of these compounds. Fenofibrate (0.5% (w/w), ±0.5 g/kg/day) induced FATP and ACS mRNA in rat liver (Fig. 4, *A* and *B*), confirming our previous observations (26). By contrast, treatment with fenofibrate did not change FATP and ACS mRNA levels significantly in adipose tissue, skeletal muscle, or heart (Fig. 4, *A* and *B*). Administration of 5 mg/kg/day of BRL 49653 was associated with the expected decrease in serum triglyceride levels (from 167 to 88 mg/dl). Furthermore, this treatment with BRL 49653 resulted in a significant induction of adipose tissue FATP (7-fold) and ACS (7-fold) mRNA levels (Fig. 4). This induction of FATP and ACS mRNA by BRL 49653 was observed in epididymal (Fig. 5) and omental (data not shown) adipose tissue. In perirenal adipose tissue, however, only FATP but not ACS mRNA was induced (Fig. 5). We observed a 1.6- and 3.1-fold induction of respective levels of FATP and ACS mRNA in skeletal muscle after BRL 49653 administration. BRL 49653 did not significantly influence the expression of FATP or ACS in liver, whereas FATP mRNA levels showed a tendency to decrease in the heart after BRL 49653 treatment.

The Induction of FATP and ACS Expression by Fenofibrate Is at the Transcriptional Level—To analyze whether the induction of FATP and ACS mRNAs occurred at the transcriptional level, a nuclear run-on assay was performed on liver nuclei obtained from fenofibrate-treated rats (Fig. 6). In comparison with control liver nuclei, the rate of FATP and ACS transcription was respectively 3- and 3.5-fold higher in nuclei from fenofibrate-treated animals. The transcription rate of acyl-CoA oxidase, a key enzyme in the peroxisomal β oxidation pathway, a positive control for fibrate action, was induced (5-fold), whereas the glyceraldehyde phosphate dehydrogenase gene, a negative control, did not change.

BRL 49653 Induces FATP mRNA Specifically in Preadipocyte Cells, whereas Fibrates Induce FATP mRNA in Cells of Hepatic Origin—To study the cellular mechanism of this induction, we investigated the regulation of the *FATP* gene expression by fibrates and BRL 49653 in hepatocyte (Fig. 7), adipocyte (Fig. 8), and muscle cells cell lines. FATP and ACS mRNA were measured in mouse AML-12 and rat Fa 32 liver-derived cell lines. A strong induction of expression of both FATP and ACS mRNA levels was seen after treatment of these liver-derived lines with fenofibric acid. Fenofibric acid induced both mRNAs optimally within 24 h (Fig. 7*A*) at a dose of 250 μM (Fig. 7*B*). The results of dose response and time course of FATP and ACS induction after treatment with fibrates seem to show an apparent difference between Fa 32 and AML-12 cells. The reason for this apparent difference in induction of FATP and ACS in the two cell lines is most likely caused by the difference in basal levels of FATP, which in Fa 32 cells is barely detectable. In contrast, under basal conditions, AML-12 expresses

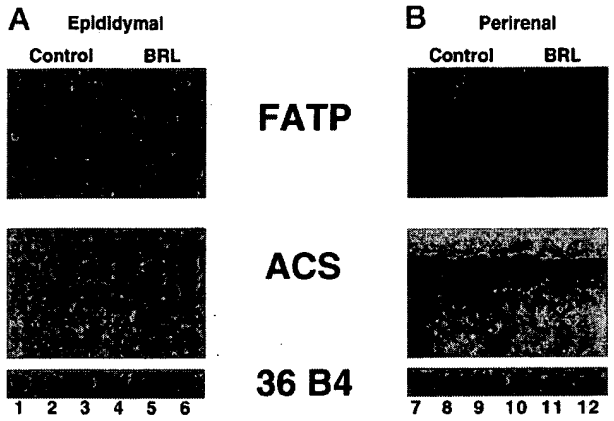

FIG. 5. **BRL 49653 induces FATP and ACS mRNA in different adipose tissue depots.** Expression of ACS and FATP mRNA in epididymal (*A*), and perirenal (*B*) adipose tissue of animals treated with BRL 49653 (5 mg/kg/day during 7 days). The blots were stripped and rehybridized with the human acidic ribosomal phosphoprotein 36B4 control cDNA. Animal treatment and preparation and analysis of RNA is described under "Experimental Procedures."

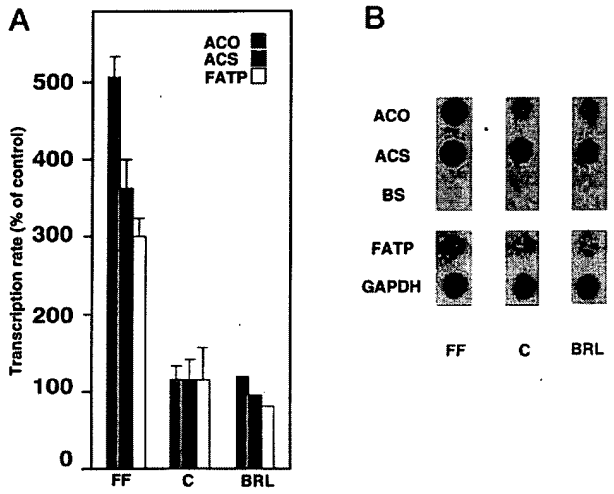

FIG. 6. **The induction of FATP and ACS by fibrates is at the transcriptional level.** Transcription rates were determined for the FATP, ACS, acyl-coA oxidase (*ACO*) and glyceraldehyde-3-phosphate dehydrogenase genes in rat liver nuclei obtained from control, or BRL 49653 (*BRL*)- or fenofibrate-treated rats (*FF*). A pUC-20 template was used as a control (*C*). Densitometric scanning of the results is depicted at the *left panel*. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase, is used for relative values; *ACO*, acyl-CoA oxidase; *BS*, BlueScript.

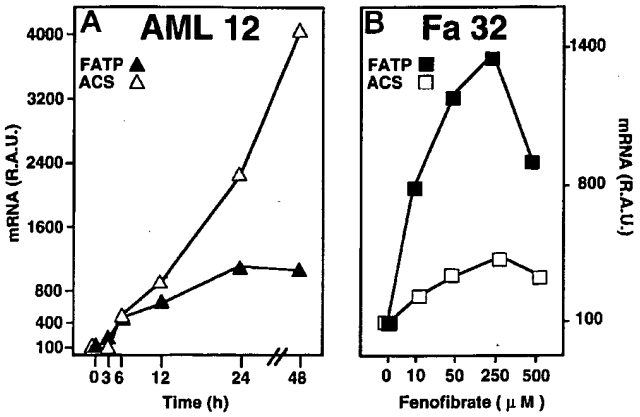

FIG. 7. **Regulation of FATP and ACS mRNA expression in the liver-derived cell lines AML-12 hepatocytes (*A*, rat) and Fa 32 (*B*, mouse) by fibrates.** *A*, AML-12 hepatocytes. Time course of FATP and ACS mRNA induction in AML-12 cells treated with fenofibric acid (250 μM). A probe for 36B4 was used as a control. *R.A.U.*, relative absorbance units. *B*, Fa 32 hepatoma cells. Dose reponse of FATP and ACS mRNA induction in Fa 32 hepatoma cells treated for 24 h with the indicated concentrations of fenofibric acid. Cells were grown and mRNA analysis was performed as described under "Experimental Procedures."

much higher levels of FATP transcript. This difference between these cells will result in an overestimation of the induction of the FATP in Fa 32 cells, explaining the discordance between relative levels of induction between FATP and ACS in the two cell lines.

To examine FATP and ACS regulation in adipocyte-like cell lines, 3T3-L1 preadipocyte cells were used. First, we analyzed the effects of BRL 49653 on nondifferentiated 3T3-L1 preadipocyte cells. As shown in Fig. 8*A*, a limited effect of BRL 49653 was observed in undifferentiated 3T3-L1 cells. In differentiated 3T3-L1 adipocytes, FATP and ACS mRNA levels were induced 5- and 9-fold after 4 days of treatment with BRL 49653. The ob 1771 preadipocyte cell line (22) was also analyzed (Fig. 8). The addition of BRL 49653 also induced FATP and ACS mRNA levels in this cell line (Fig. 8), whereas fenofibric acid had only a weak effect (data not shown).

Finally the effects of both BRL and fenofibric on L6 muscle cells were analyzed. Unlike in adipocyte or hepatocyte cell lines, no change in L6 ACS and FATP mRNA levels were detected upon treatment with either fenofibrate or BRL 49653 (data not shown).

Induction of FATP or ACS mRNA Levels Results in a Change in FA Uptake into Cells—To verify whether changes in mRNA levels of FATP and ACS were correlated with alterations in fatty acid uptake, we analyzed [^{14}C]oleic acid uptake in Fa 32 and AML-12 hepatic cells, L6 muscle cells, and 3T3-L1 preadipocytes. As shown in Fig. 9, fatty acid uptake of [^{14}C]oleic acid significantly increased after treatment of the liver-derived AML-12 cells with fenofibric acid and after treatment of the differentiated adipocyte-like 3T3-L1 cells with BRL 49653. In Fa 32 cells, fatty acid uptake was also increased (data not shown). The increase, although statistically significant, was however less pronounced than in AML-12 cells. As expected in view of the absence of a major regulation of ACS and FATP mRNA in muscle cells, no effects of either fenofibric acid nor BRL 49653 were observed on FA uptake in L6 muscle cells. The regulation of FA uptake was hence completely consistent with the regulation of respective mRNA levels of FATP and ACS in the various cell models.

DISCUSSION

Both ACS and FATP have been suggested to play a crucial role in the transport of fatty acids into the cell (4). FATP acts as a fatty acid transport protein, whereas ACS prevents efflux of the newly imported fatty acids by their esterification with coenzyme A. Fatty acids are important cellular components that can function both as metabolic substrates or as signaling molecules, by functioning as second messengers and triggering signal transduction pathways or by directly activating transcription factors such as the PPAR family of nuclear receptors. Since FATP and ACS control, in part, the intracellular availability of FAs, important PPAR activators, the aim of the present investigation was to perform detailed analysis of FATP and ACS expression and to establish whether FATP and ACS expression themselves might be subject to control by PPARs.

In the liver, one of the major organs susceptible to peroxisomal proliferation, *FATP* gene transcription is strongly induced upon fibrate treatment. This strong induction is not surprising if one takes the strong induction of peroxisomal β oxidation into account after fibrate treatment. FATP is likely to be responsible in part for the increased FA import necessary to sustain

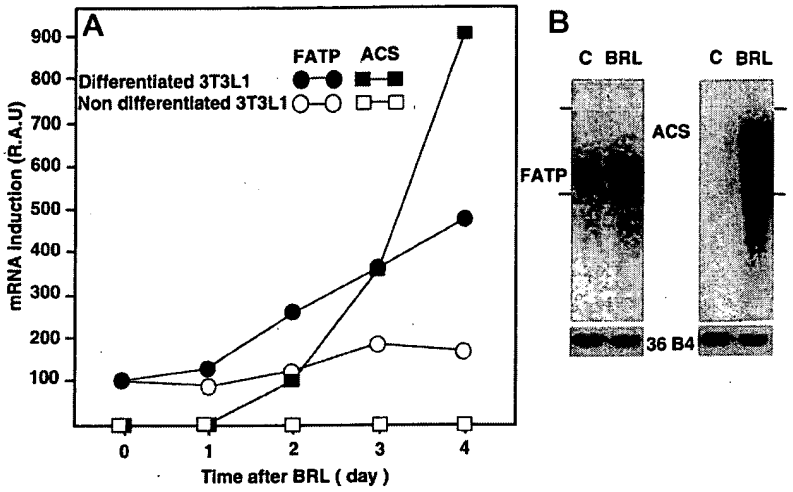

FIG. 8. **Regulation of FATP and ACS mRNA expression in 3T3-L1 preadipocytes (*A*) and in ob 1771 preadipocytes (*B*) by thiazolidinediones.** *A*, 3T3-L1 cells. Differentiated or undifferentiated 3T3-L1 cells were treated with BRL 49653 (10 μM) for different time periods. RNA was extracted and analyzed for FATP and ACS expression as described under "Experimental Procedures." A probe for 36B4 was used as a control to normalize the results. *R.A.U.*, relative absorbance units. *B*, ob 1771 cells. Differentiated ob 1771 cells were challenged during 3 days with BRL 49653 (10 μM) and FATP, and ACS mRNA levels were analyzed.

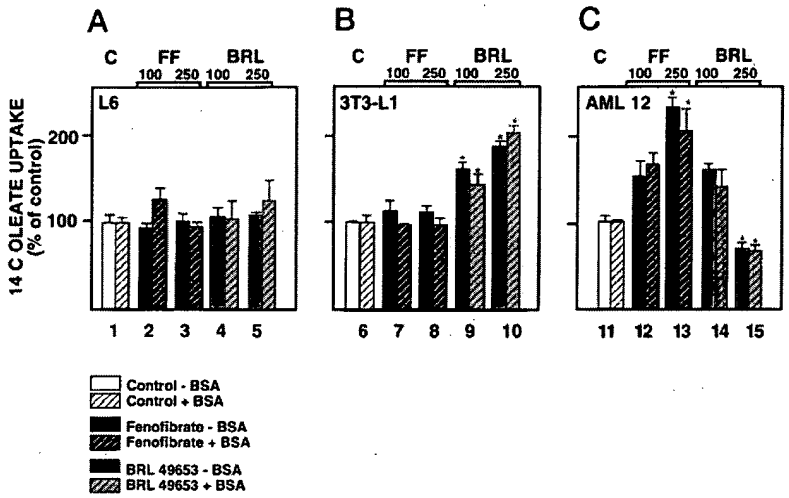

FIG. 9. **Fatty acid uptake is increased in parallel with the changes in FATP and ACS mRNA levels.** [^{14}C]Oleic acid uptake in L6 muscle cells, 3T3-L1 preadipocytes, and AML-12 hepatic cells is shown. Cells were untreated or incubated with either 100 or 250 μM fenofibric acid (*FF*) or 100 or 250 nM BRL 49653 for 24 h. [^{14}C]Oleic acid uptake was then performed as described under "Experimental Procedures." It is indicated whether cells were washed without (−BSA) or with BSA (+BSA).

this increased β oxidation. Furthermore, a striking parallelism exists between the induction by fibrates of a number of genes involved in fatty acid import in the liver. In fact, the mRNAs for lipoprotein lipase (LPL) (27), ACS (this paper and Refs. 27 and 28), and *FATP* genes are all induced after fibrate treatment in the liver. This coinduction of genes seems to prime the cells for more efficient β oxidation. Induced liver LPL expression will increase lipolysis in the vascular bed of the liver, generating more fatty acids, which are then avidly taken up by the cells thanks to the higher levels of FATP expression. Efflux of these imported fatty acids is prevented by induced ACS levels, which in addition, primes them for subsequent metabolism. Therefore it seems that fibrates not only induce β oxidation but also induce genes important for supplying the cells with the extra fatty acids they need to sustain this increase in β oxidation.

In the intestine, FATP was also induced by fibrates, albeit to a lesser extent. The FATP induction in this tissue shows a striking parallel to the induction of CD36 after fibrate treatment in this tissue (29). In contrast to the liver and intestine, heart FATP mRNA levels did not vary substantially under fibrate treatment. The basal levels of FATP expression were, however, very high in heart, which almost exclusively uses fatty acids as energy source. In this tissue, fatty acids are, however, constitutively metabolized to provide energy necessary for the contraction of the heart muscle. Unresponsiveness of FATP expression in the heart to hormonal control could hence be physiologically significant, since the continued function of the heart is far too crucial to allow any form of major regulation of a transporter vital to its energy supply. This would suggest that in the heart, the FATP promoter is maximally active, resulting in a high level of constitutive FATP gene expression, which would be consistent with the high basal levels of FATP mRNA in this tissue. Similar to the heart, less extreme changes were observed in adipose tissue FATP mRNA after fibrate treatment. The absence of an effect of fibrates on FATP expression in adipose tissue is most likely due to the lower levels of PPARα relative to PPARγ. For kidney and intestine, the regulation of ACS and FATP by fibrates is discordant. This is consistant with the less crucial functions lipids play in kidney and intestinal metabolism. Kidney expressed only low levels of FATP mRNA, and its expression was furthermore refractory to induction by fibrates. Relative to heart and liver, the kidney utilizes relatively little fatty acids, and therefore a coordinate import mechanism is of lesser importance. In intestine, fatty acids are primarily absorbed, but they are less actively metabolized than in heart and liver. Therefore, intestine apparently has an actively regulated transport mechanism, as evidenced by regulation of the expression of both FATP and FAT, another transport protein that is also expressed and highly regulated in this organ (29). Since fatty acids are less actively metabolized and rather resecreted under the form of lipoproteins, there is less need for their conversion to acyl-CoA derivatives and hence less need for coordinated regulation of ACS together with these transport proteins suggested to be implicated in fatty acid transport.

The demonstration of the inducibility of the FATP and ACS

genes by PPARγ ligands such as the thiazolidinedione BRL 49653 has important implications for adipocyte physiology. PPARγ has been shown to promote preadipocyte determination as well as terminal differentiation (13, 30), and its mRNA is itself induced in the earliest steps of adipocyte differentiation before the induction of early marker genes for adipocyte differentiation. Many of these genes induced during adipocyte differentiation encode proteins involved in lipid storage and metabolism. The increase in FATP and ACS expression in differentiated adipocyte-like cells caused by PPARγ ligands will result in an increased delivery of fatty acids to the adipocytes, which possibly sustains a positive regulatory feedback loop involving continued PPARγ activation of the FATP and ACS (28) genes and aimed at promoting and maintaining the mature adipocyte phenotype. In fact, in addition to the thiazolidinediones, certain fatty acid-derived prostaglandin derivatives, whose delivery to the cell is increased by FATP, bind to and/or activate PPARγ (19, 20, 31). This hypothesis is supported by the observation that fatty acids (including arachidonic acid-derived prostaglandins) and fatty acid analogues induce the expression of adipocyte-specific genes and enhance adipocyte conversion (30, 32–35). In addition to being potent PPAR activators (7, 12, 31, 36, 37), fatty acids will provide the necessary building blocks for triglyceride accumulation, ultimately enhancing adipocyte differentiation. The PPAR-mediated activation of FATP and ACS expression in cells of the adipogenic lineage might furthermore in part be responsible for the previously reported capacities of thiazolidinediones to induce adipocyte differentiation and induce the development of obesity (38–46). In this context, it is interesting to note that the PPARγ-mediated effects of BRL 49653 on FATP and ACS expression might act in concert with induced LPL expression and the reduced leptin mRNA and protein levels and the associated increase in caloric intake enhancing energy storage in the adipocytes observed with this compound (47, 48). Interestingly, FATP and ACS are not coordinately regulated in perirenal and epididymal adipose tissue stores. This differential regulation is consistent with the distinct metabolic nature of the different adipose tissue depots (49–51). Further studies are required to determine whether the role of FATP is a consequence of or is causative of the physiologic differences between the adipose tissue depots.

The tissue-selective effects of the various PPAR activators/ligands are highly intriguing and provide insight in their effects on triglyceride metabolism. Fibrate treatment induced FATP and ACS expression strongest in liver, whereas BRL 49653 had no effect on liver, but strongly induced adipocyte FATP and to a lesser extent ACS expression. The effects of fibrates (PPARα activators) on the liver and PPARγ ligands in adipose tissue correlates well with the tissue-specific expression of the respective receptors and suggests that the FATP and ACS genes show a tissue-selective activation similar to the one previously described for the *LPL* gene (27). In this context, we need however to address the discrepancy between ACS and FATP regulation after BRL 49653 administration in skeletal muscle. Muscle tissue expresses very low levels of PPARγ,[2] which is consistent with the absence of an important regulatory effect of PPARγ activators in this tissue as observed for the LPL (27) or FATP (this study) genes. In this context, the induction of ACS expression by BRL 49653 is however difficult to explain. One must however bear in mind that not all of the effects of the thiazolidinediones are mediated via PPARγ activation, and it is has been shown that these agents activate several other signaling pathways (52–54). Further investigations need to address whether ACS expression, unlike FATP or LPL expression, is subject to such a regulatory circuit.

One remaining question is the relationship between PPARγ, thiazolidinediones, and insulin resistance. It is tempting to speculate that the increase of LPL, ACS, and FATP activity in adipose tissue is related to the antidiabetic effects of the thiazolidinediones. Due to the enhanced triglyceride clearance in adipose tissue, less triglycerides will become available to be hydrolyzed to fatty acids in the vascular bed of the muscle. Furthermore, relative to the strong induction of FATP and ACS in adipose tissue by BRL 49653, very limited inductions of both genes are observed in skeletal and heart muscle, favoring uptake of fatty acids in adipose tissue relative to muscle. In view of the inhibitory effects of fatty acids on insulin-mediated glucose metabolism (55), the decrease in fatty acids delivered to the muscle cells might be responsible for the improvement in insulin sensitivity of this tissue.

In conclusion, FATP and ACS mRNA levels can be regulated in a tissue-specific fashion by PPARα activators and PPARγ ligands. In adipose tissue, the increase in FATP, ACS, and LPL (27) production after treatment with thiazolidinediones will enhance the clearance of plasma triglycerides (27, 56) and provide the (pre)adipocytes with additional fatty acids, which can further stimulate the transactivation capacity of PPAR or which can be stored under form of triglycerides. In the liver, the enhanced production of FATP and ACS after fibrates together with the increase in β oxidation and the reduced production of apoCIII (57), may contribute to the hypolipidemic action of these compounds. This tissue-selective induction of FATP and ACS gene transcription by activators of different PPARs, demonstrates the feasibility of the development of highly specific PPAR subtype-specific agonists and antagonists, which can be used as drugs.

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